High-Throughput Screening System for Determination of Estrogenic Properties of Small Molecules

Undergraduate Honors Research Thesis

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Abstract

Estrogenic compounds have important pharmaceutical and toxicological effects on humans. Pharmaceutically, estrogens can be used to treat diseases such as breast cancer. Toxicologically, estrogens are known to affect the growth and development of young girls. Understanding the estrogenic properties of a target compound would allow for compounds to be assessed as pharmaceuticals and as environmental contaminants.

Current methods for measuring estrogenicity involve the use of animal models. A model organism is grown, treated with the test compound, sacrificed and autopsied. For the model to provide significant data, the amount of animal models used is significant. The amount of animals needed to conduct a high-throughput screening is time prohibitive and economically unviable.

In this work, a high-throughput screening system for determining the estrogenicity of a compound is proposed. This method uses a human estrogen receptor beta and a thymidylate synthase reporter system in *Escherichia coli*, in order to relate the growth of cells to the concentration and estrogenicity of a compound. The biological, robotic and data processing aspects of the assay were validated initially. Once validated, this system was used to test thirty compounds. The high-throughput screening system could distinguish between estrogen agonists and non-agonists. The system determined that 7 compounds in the test library were estrogen agonists.

Acknowledgements

I would like to thank Dr. David Wood for giving me the opportunity to do research in his laboratory for the past year. I would also like to thank Brian Saunders for his initial insights. I would also like to thank Jeevan Baretto for providing me with the bacterial strains I used. Finally, I would like to thank Angela Chen and Lily Glick for their assistance in culturing bacteria and in preparing media.

Table of Contents

List of Figures

List of Tables

Introduction

Estrogens are hormones or hormonal compounds that function in many critical metabolic pathways in the human body [1]. Estrogen compounds have several important roles in human development, including roles in sexual differentiation and roles in the onset of puberty. The presence of estrogen *in vivo* is regulated by the endocrine system, which uses the hypothalamus, the pituitary gland and the gonads to control the concentration of estrogen in the body through all stages of development. Estrogens plays important roles in several important diseases, including hypospadias, testicular cancer, breast cancer, and prostate cancer.

When estrogen-like compounds enter the human body, they can affect the function of estrogen pathways [2]. These compounds interfere with estrogen pathways in multiple ways: by altering normal estrogen transportation, by interfering with estrogen synthesis, or by competitively binding with native estrogen hormone receptors. Estrogen-like compounds can affect the normal growth and development of humans, either by altering the proper expression of secondary sex characteristics or by altering the developmental cycle of humans.

Estrogen-like compounds have important function in the treatment of diseases such as breast cancer [3]. Because estrogen-like compounds can bind to hormone receptors, they can have a pharmacological effect on tissues which express the estrogen hormone receptor. Therefore, compounds can be screened for their estrogenic properties to determine potential hits for novel pharmaceutical compounds.

Some environmental compounds are known estrogen-like compounds or are suspected to be estrogen-like compounds [4]. Two compounds of this nature are dichlorodiphenyl-trichloroethane (DDT) and bisphenol A (BPA). DDT, a commonly used pesticide that is

currently banned, has been shown to possess estrogenic properties, while some of DDT's breakdown products possess estrogenic or androgenic properties [8]. Bisphenol A has been shown in several assays to have estrogenic properties, but the extent of these qualities is highly controversial. Estrogen-like compound levels in drinking water has been attributed to a decreasing age in puberty onset in prepubescent girls [4].

The estrogenic properties of a compound cannot be easily determined. It is not possible to predict the hormonal activity of a novel compound from inspecting its chemical structure. Figure 1 below shows the chemical structure of estradiol, the human estrogen hormone, DDT and bisphenol A. Despite all three compounds possessing estrogenic properties, their chemical structures vary greatly.

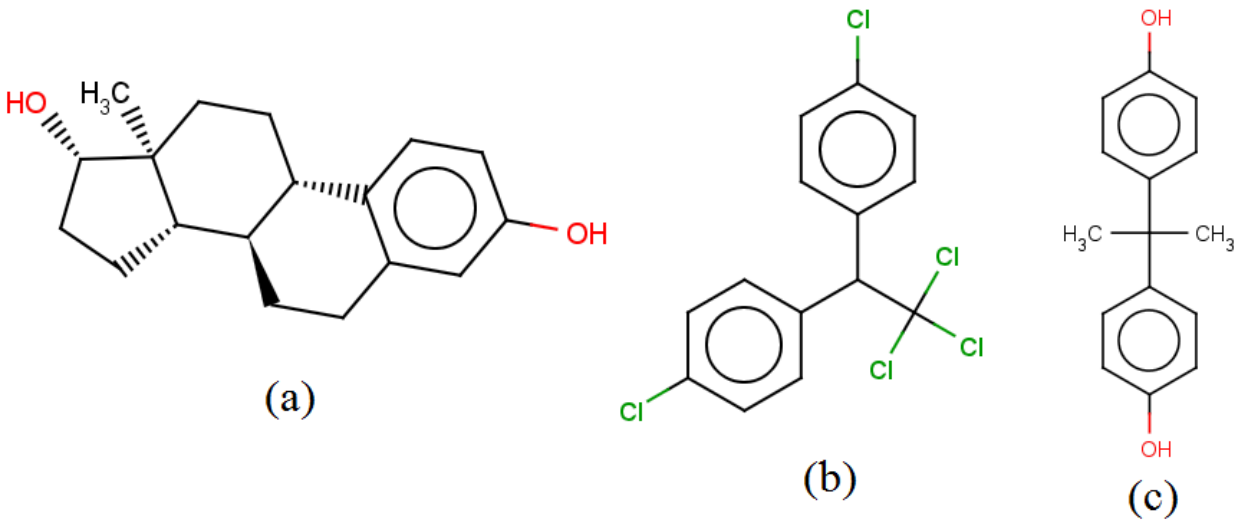

Figure 1: Representative Estrogenic Compounds

Animal models are typically used to assess the estrogenic properties of a compound [4]. One animal model frequently used is the rat [5]. Two *in vivo* tests for estrogenic properties of molecules with rat models involve measuring the uterine wet weight and measuring vaginal epithelial cell cornification [6]. Rats are raised and treated with the compound of interest. When

the rats reach maturity, they are sacrificed and autopsied to measure the property of interest. These methods produce false positive results with non-estrogenic compounds such as testosterone and fail to produce positive results with weaker estrogenic compounds. Another animal model used to determine the estrogenic properties of a compound is rainbow trout [7]. Rainbow trout are grown for 21 days with the compound of interest. They are then sacrificed and autopsied to measure their weight and plasma vitellogenin concentration. This method is highly dependent on the growth of the fish.

In addition to the scientific limitations of these methods, there are practical limitations which prevent the adaptation of these methods to high-throughput screening. First, a large inventory of laboratory test animals must be kept and maintained. These animals must be the proper age and weight in order to use them in testing. The animals must also be kept at consistent conditions, in order to control for the effect of food and living conditions on the response [6]. The costs of maintaining these animals at the proper condition for testing can be significant. In addition, for the testing to yield significant and precise results, large sample sizes must be used [6,7]. Using large sample sizes increases the cost greatly.

Second, animal research requires written proposals and approval from an institutional review board. Requesting approval for testing a large quantity of potentially estrogenic compound in an initial screening experiment would be very time-consuming to perform. The financial costs of keeping laboratory animals and the time cost of seeking regulatory approval significantly hinder the potential of using laboratory animals in high-throughput estrogenic screening applications.

Background

In order to supplement estrogen testing methods and reduce the need for animal models in preliminary estrogen testing, additional testing methods are needed. The method needs to show a quick response to environmental estrogens, show a difference in response to strong and weak estrogens, and show an easily measurable, quantifiable response. Bacterial cells grow quickly and grow in response to their environment. Therefore, bacterial sensor cells (biosensors) that sense environmental estrogens and grow in to response to them would have the necessary properties to measure the estrogenic properties of compounds quickly and efficiently. Biosensors have been proposed as a supplementary tool to animal models for compound testing [9]. By coupling the growth of bacteria with the receptor phenotype, the biosensor only demonstrates growth and activity when it is exposed to an estrogenic compound.

Biosensor Mechanism of Action

The biosensor was constructed using *Escherichia coli* D1210ΔthyA, a bacterial knockout strain which lacks native thymidylate synthase (TS) enzyme function [9]. Without TS enzyme function, the knockout strain cannot synthesize new DNA and is unable to grow in minimal media. Thymidylate synthase catalyzes the reaction of 5,10-methenyltetrahydrofolate (MTHF) and deoxyuridine monophosphate (dUMP) to form dihydrofolate (DHF) and thymidine monophosphate (dTMP) [10]. Figure 2 on the next page shows the thymidylate synthesis reaction cycle [11]. Without TS enzyme, DNA cannot be synthesized, which prevents cell replication. In rich media supplemented with thymine, the production of dTMP using the TS enzyme is not needed, because the cell can combine thymine directly with deoxyribose to create dTMP. In minimal media lacking thymine, the cells are unable to synthesize DNA and are unable to perform cell division.

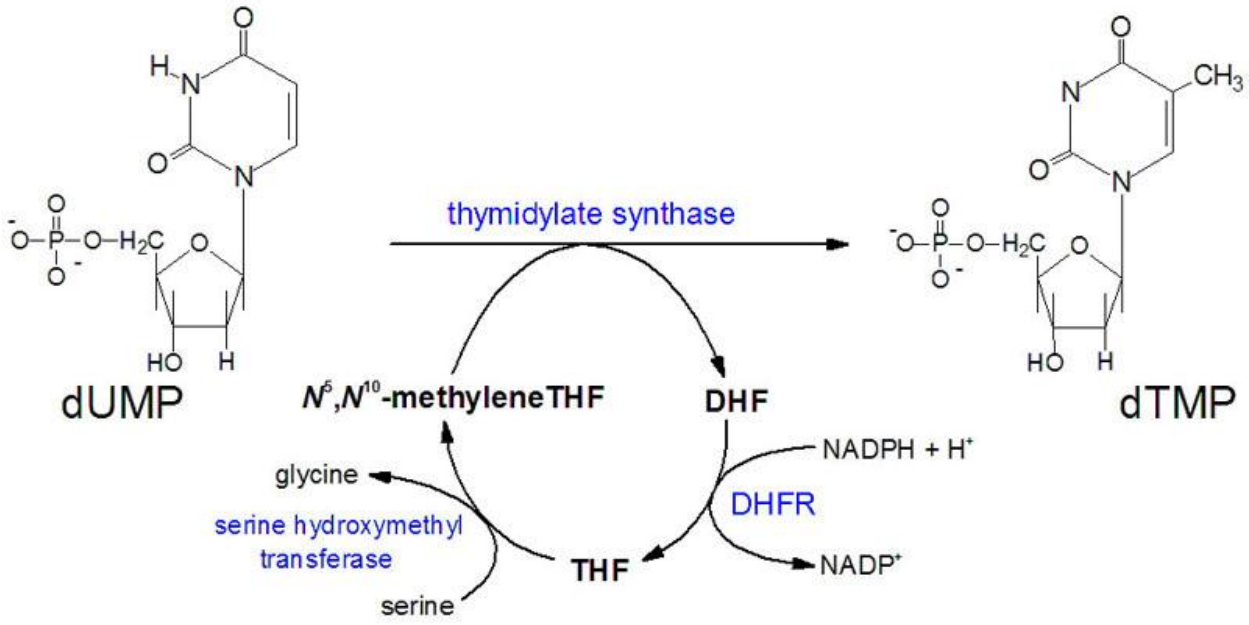

Figure 2: Thymidylate synthase reaction cycle [11]

Intein Mechanism of Action

Inteins are self-excising protein segments that are found in cells and viruses [12]. Naturally, inteins self-catalyze and remove themselves from a longer protein segment. In addition, they connect the segment attached to the carbon side (c-terminus) and the segment attached to the nitrogen side (n-terminus) with a peptide bond. This creates a smaller protein segment. Inteins function similarly to regulatory proteins, which change their splicing pattern in response to the environment [13].

Inteins are important tools in biotechnology [12]. By engineering the natural properties of wild-type inteins, proteins can be engineered to splice in response to specific environmental conditions, such as temperature, concentration or conformation. Inteins can be engineered to alter their splicing patterns, allowing splicing at only one terminus or at neither terminus. One application of inteins in bioengineering is protein purification. Proteins can be engineered to

contain purification tags that cleave at one terminus after purification, leaving only the desired protein. In the biosensor application, the intein was used to couple the activity of TS to the human estrogen receptor beta (ERβ) hormone receptor.

Biosensor Construction

After selecting the initial knockout strain, a gene had to be constructed to introduce the TS enzyme and the hormone receptor to the cells. A schematic of the completed gene, pMIT::ERβ*, and the resulting protein is shown on the next page in figure 3. In constructing the gene of interest, several issues had to be resolved to allow for proper expression of the final protein [14]. First, the receptor could face issues with proper folding. When a protein is synthesized in a non-native host, its ability to properly fold can be compromised, due to differences in the cell's microenvironment. Small changes in cytoplasmic concentrations can prevent the proper folding of the receptor, altering its function. Second, the protein of interest may not be soluble in the new host. Again, this is a result of differences between the new host's cytoplasmic environment is the native host's cytoplasmic environment. Third, the yield of the protein may be high enough to allow for a proper response.

In order to assist the protein in properly folding, a gene fusion was used. The gene fusion connects the gene of interest with the intein. By inserting the gene in between two segments of the intein gene, the target protein will be assisted in folding by the intein [14]. In order to assist in the solubility of the protein, a maltose binding protein (MBP) tag was used. MBP is a very soluble protein. By fusing the MBP to the intein system, the solubility of the protein increases. In addition to increasing the solubility of the target protein, the MBP fusion also increases the activity of the protein [14].

For this cell, the RecA intein from *Mycobacterium tuberculosis* was selected. In between the c-terminus and n-terminus segments of the intein, the human ERβ receptor gene is inserted. The TS enzyme was added to the c-terminus of the RecA intein. MBP was added before the n-terminus of the intein. Two important amino acids were changed in the gene of interest. The initial cysteine in the n-terminus segment was changed to alanine, and the final asparagine in the c-terminus segment was changed to alanine. These mutations prevent the intein from splicing and altering the DNA. After these mutations, the protein allows the binding of an estrogenic compound to the human ERβ receptor to increase the activity of the TS enzyme. Finally, a tac promoter was used to facilitate transcription of the gene in the *E. coli* cells.

Figure 3: Protein fusion of estrogen receptor and TS [14]

Biosensor Response to Compounds

The cells show a dose-response relationship with the presence of estrogenic compounds. Figure 4 below shows a representative dose-response curve using the biosensors and a human estrogen receptor agonist [14]. When the logarithmic concentration of the estrogenic compound

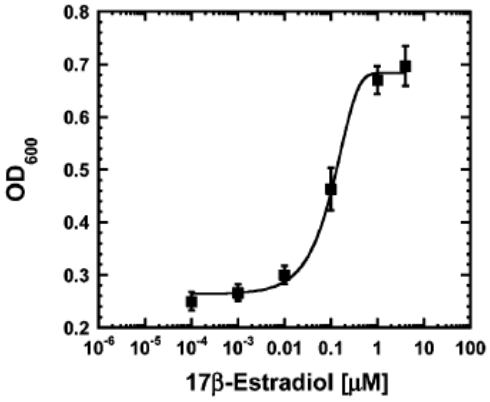

Figure 4: Representative dose-response curve [14]

added to the cells is compared to the growth of cells, a sigmoidal curve is present. Because the concentration is related to the probability of a compound-receptor interaction, the response from the cells will not be linear [15]. From the sigmoidal curve, the estimated concentration at which 50 percent of the receptors respond (EC50) can be determined. By comparing the EC50 of compounds, the agonistic effect of the compounds on human ERβ receptors can be compared.

Objective

The main objective of this work was to create and validate an assay which could detect the estrogenic properties of a small molecule without the use of animal testing. This system needed to be able to reproduce known estrogenic properties of compounds and measure the estrogenic properties of uncharacterized compounds. In addition, the assay needed to conform to the requirements of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The ICCVAM has established stringent requirements for any non-animal assay to be considered an alternative to animal testing [16]. These requirements include experimental design considerations and chemical considerations.

In addition to stringent assay requirements, the ICCVAM requires the testing of many reference compounds. These reference compounds contain strong agonists, weak agonist and antagonists. Many compounds are pharmaceuticals, while some are commonly used environmental compounds such as pesticides. A list of the compounds required by the ICCVAM for testing and their expected behavior are included in appendix A. Some of the compounds that were listed by the ICCVAM as test compounds were not tested, however. Many of the compounds, due to their hormonal and pharmacological properties, are controlled substances,

which could not be easily purchased. Other compounds were unable to be shipped, due to their hazardous nature. Because of this, only a select number of compounds could be tested.

Methods

The validation and testing of the assay can be divided into four main phases. In the first phase, the biological conditions of the assay were developed. This includes the growth media, the amount of times for each growth phase and the inoculation amounts. In the second phase, the liquid handling robots were validated. This was accomplished by writing and testing calibration programs, which could verify the amount of liquid that was pipetted in each step. In the third phase, the plate reader was verified for its accuracy and precision. Next, the data processing programs had to be modified to allow for higher throughput processing. Once the validation was complete, the target compounds were tested in the assay.

Biological Validation

Initially, the ERβ biosensors were grown on Petri dishes for 18 hours at 37 degrees Celsius. The plates were made using Luria-Bertani broth agar (LB agar) fortified with ampicillin and thymine. Specific recipes for the media used are located in appendix B. LB agar is a rich, undefined media that provides the cells with a carbon and nitrogen source, allowing the cells to grow. The ampicillin is added to select for cells that keep their plasmid during replication. Without selective forces keeping evolutionary pressure on maintaining the plasmid, the cells can spontaneously lose the plasmid and lose the ERβ hormone receptor and TS enzyme. Thymine is added to assure that the cells can grow in the media without risking thymineless death, a rapid cell death process that is caused by a lack of thymine for DNA synthesis.

After the growth on plates, the cells were grown in liquid media at 37 degrees Celsius for between 6 and 12 hours. The liquid media contained Luria-Bertani broth (LB media), thymine and ampicillin. The LB media is a rich media, similar to LB agar, which provides a carbon and nitrogen source to the bacteria. The ampicillin provides selective pressure towards the cells keeping their plasmid, and the thymine assures that the cells can grow in the media. To seed the liquid media, a single colony was used. By using a single colony, the homogeneity of the cells seeding the liquid culture can be assured. A single colony on a petri dish must arise from a single cell, so by selecting a single colony, the cells must be a single strain.

The liquid cells were grown until they reached the late growth phase. This assured that the cells were in high concentration, but that no cells had begun to die. Figure 5 below shows a representative bacterial growth curve. As soon as the bacteria reach the stationary phase, the

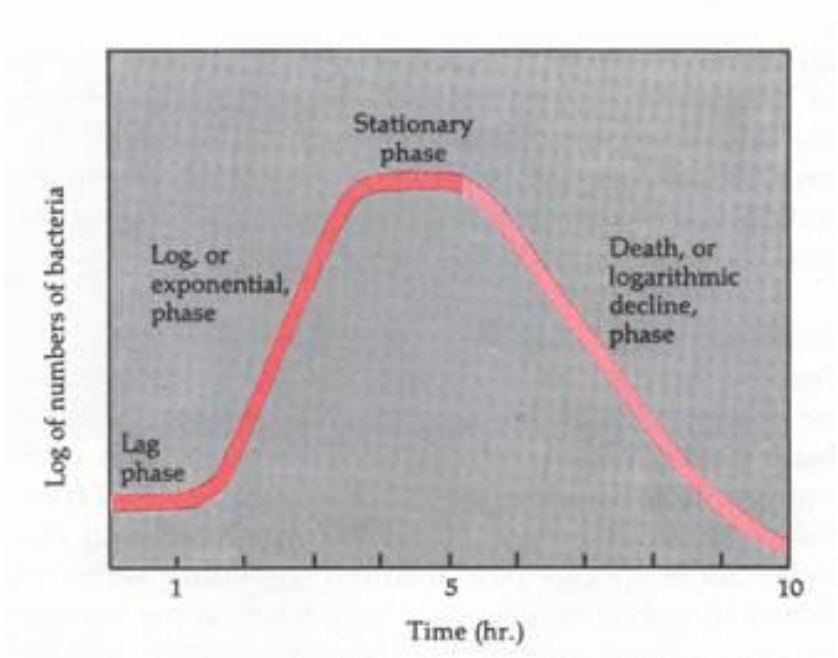

Figure 5: Representative bacterial growth curve [18]

cells begin to die. Transferring dead cells is not desired, because dead cells will affect the optical density reading but not respond to estrogenic compounds. To determine the current growth phase of the cells, the optical density (OD) of the cells was determined. The OD reading was done at 600 nanometers. 600 nanometers is typically used to measure *E. coli* growth in LB broth because no components of the media absorb light at that wavelength [17]. In addition, the OD at this wavelength is proportional to the scattering of light by the bacteria, which is directly proportional to the number of cells in the broth.

The cells were then transferred to thymine free media (-Thy). –Thy is a chemically defined, minimal media that contains specific quantities of chemical supplements necessary for growth. Other types of growth media, such as LB media, provide inconsistent amounts of a wide variety of chemical supplements. The specific nature of rich chemical components is impossible to determine, because the components are produced by incomplete digestion reactions that break chemicals down in an uneven manner. Although LB media components are less expensive and provide trace components which cells may need for growth, they are not preferred for the final growth phase. In the final phase, the exact composition of the media must be known, in order to assure the only component affecting the ERβ hormone receptor is the estrogenic compound. Also, no thymine can be present in the final growth media. Without thymine, cell growth can only occur if the ERβ hormone receptor is bound and TS is released.

The LB media with cell growth is added to –Thy media. A ratio of 1 to 200 is used to add the cells. This ratio assures that enough cells are added to observe a response, but that the concentrations of other LB media components are minimal. The –Thy media with cells is then pipetted into 96-well plates. The compounds of interest are also added to –Thy media in the plates. The compounds of interest are added as a dimethyl sulfoxide (DMSO) solution. To

avoid the toxic effect of DMSO on the cells, the solution is added such that the final concentration of DMSO in the cell solution is one percent [19].

Once a 96-well plate is prepared, it is incubated at 34 degrees Celsius. To determine the ability for cells to grow in the plates, the growth of cells in the presence of 17β-estradiol (E2), triiodothyronine (T3) and tiratricol (Triac) was measured. The time for an optimum dose-response curve to be observed had to be determined empirically. Initially, the optimum growth period was assumed to be between 12 and 21 hours. By measuring the OD of the bacteria in the 96-well plates at varying growth times, the growth could be correlated to the amount of growth. If the growth time was too short, the dose-response curve would not have enough points above the growth increase to assure that the fitted curve is sigmoidal. If the growth time was too long, the bacteria would grow too much, and the curves would be too flat. As a result, the dose-response curves would be less pronounced, and curve fitting would be much more difficult. Figure 6 below contains a representative dose-response curve with the desired properties.

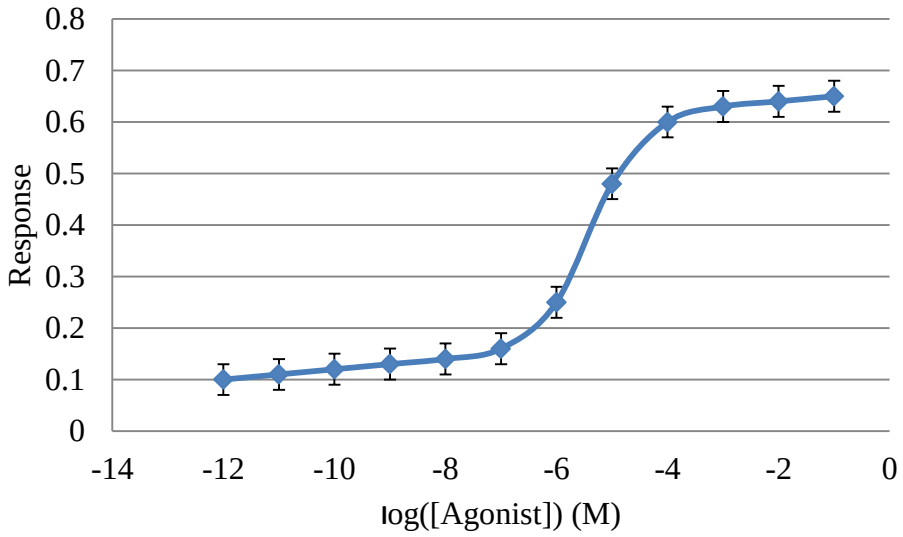

Figure 6: Representative dose-response curve

Liquid Handling Validation

Once the biological aspects of the assay were confirmed, the liquid handling robot was validated to assure that the amounts of liquid transferred by the robot were accurate. To do this, the absorbance of tartrazine dye at varying concentrations was tested. Tartrazine was selected as the dye of interest because it has a distinct absorbance peak, because it is non-toxic and because it is a commonly used calibration dye [20]. The slope of the calibration curve was used to determine if the dye was being added in precise and consistent amounts. For calibration, two liquid handling tools were considered: a 200 microliter tool and a 20 microliter tool. If the regression coefficient was indistinguishable between hand pipetting and robotic pipetting, the liquid handling robot was considered to be precise.

Absorbance Validation

To verify the precision of the plate reader, the extinction coefficient of the tartrazine was experimentally determined and compared to literature values. The absorbance of the dye can be determined using the Beer-Lambert law. Equation 1 below shows the relationship between absorbance and concentration of dye.

$$A = \varepsilon l c \tag{1}$$

In this form of the Beer-Lambert law, the absorbance is determined experimentally. The path length and concentration are known. By measuring the absorbance as a function of concentration, the extinction coefficient of the dye can be determined. The experimentally determined coefficient can be compared to the literature value to determine the accuracy of the plate reader.

Data Processing

Once the method is validated and data is collected, the data must be processed. Because each dilution plate was repeated in triplicate and each experiment used multiple dilution plates, there was a significant amount of data to process for each experiment. In order to construct dose-response curves for each estrogenic compound tested, a Matlab program was used. This program read a data file and calculated average absorbances and standard deviations of the absorbances. Next, the program fit the data to the Hill equation, an equation widely used in data modeling because of its flexibility in biological and pharmaceutical applications [21]. In this case, the Hill equation had the form of a 4-factor logistic equation, corresponding to a sigmoidal curve. Equation 2 below shows the equation used to fit the data.

$$y = a + \frac{b-a}{1+d*10^{c-x}} \quad (2)$$

In order to fit the data to the curve, non-linear regression was used to determine the four constants. In addition, the EC50 of each curve was calculated. Each plate was graphed. Appendix B contains the Matlab programs used to model and graph the data.

The program will fit all data to a sigmoidal curve, regardless of the correctness of a sigmoidal fit. Therefore, each fit was visually inspected to determine the correctness of the sigmoidal fit. Several rules were applied to determine the validity of the sigmoidal fit. First, the EC50 had to be in a concentration within the data recorded. If the program predicted an EC50 beyond the investigated concentration, the EC50 value was disregarded. Second, if the fit showed antagonistic behavior, rather than agonistic behavior, the fit was disregarded. Because the assay cannot induce antagonism, any antagonistic behavior that was fitted is the result of data fitting errors.

Results

Biological Validation Results

The initial growth of bacteria in LB agar Petri dishes was successful. Growth of individual colonies across the plate was observed. The colony morphology was consistent across the plate. Therefore, the bacteria were considered to be a single species that contained the desired plasmid.

A large variation in growth time required for the liquid broth to reach an optimal OD was observed. This challenged the consistency of the experimental protocol. The hypothesized source of this variation was the variation in colony size used to seed the liquid media. Because a single colony contains between 3,000,000 and 5,000,000 cells and because cell growth is exponential, the initial amount of bacteria seeded alters the growth kinetics greatly. To overcome this variance, an additional culture step was added. The single growth step in liquid media was replaced with two steps using the same LB media fortified with thymine and ampicillin. The first step was an overnight growth for 12 to 14 hours. After this time, cells were consistently in the stationary phase of growth. The cells were then transferred in a one to 100 dilution into fresh LB media fortified with thymine and ampicillin. By adding this step, the second culture time was between 2.5 to 3.5 hours. The additional culture step greatly increased the protocol's consistency.

The plate reader was able to successfully read plates with bacterial growth. In the presence of a 96-well plate, the plate reader could distinguish between wells without growth and wells with growth. Until the accuracy of the plate reader could be robustly determined, the reader could be used to determine the presence of a strong dose-response curve and no dose-response curve.

Dose-response curves for the biosensors were constructed after 18 hours of growth for E2, T3 and Triac. Figure 7 below shows the dose-response curve for E2. Dose-response curves for all three compounds are included in appendix C. The figure shows that a dose-response relationship exists between the human ERβ receptor beta and E2. Because E2 is the natural analog of the human ERβ receptor, the result confirms the success of the protocol. Table 1 below shows the results for all three compounds. Because T3 and Triac are thyroid hormone analogues, there should be no effect on the ERβ receptor. Because no effect is observed, the biosensor can discriminate between estrogenic and non-estrogenic compounds.

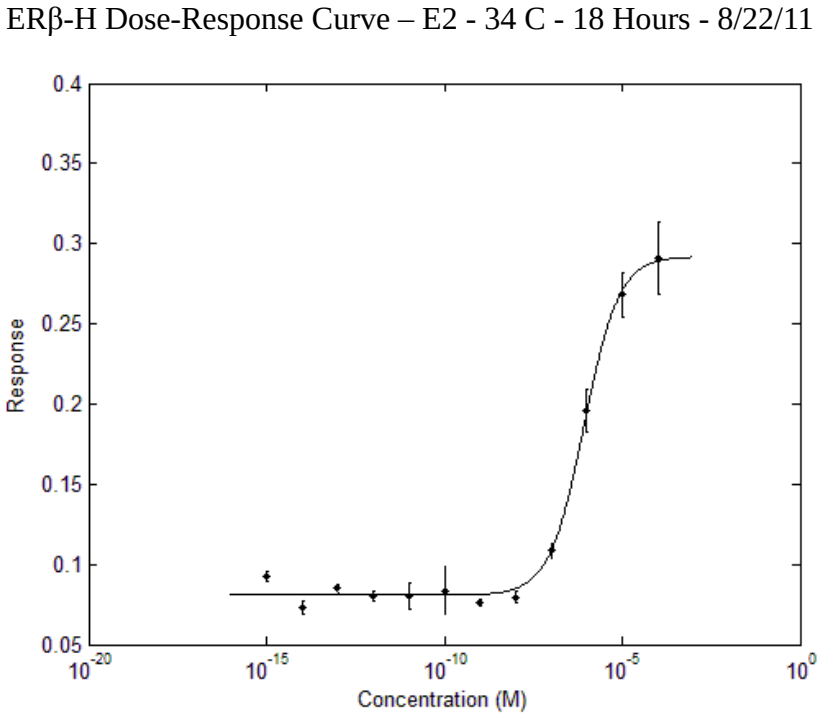

Figure 7: Dose-response curve for E2

Table 1: Dose-response curve summary

Compound	E2	T3	Triac
Does-Response Curve	Yes	No	No
Expected Relationship	Yes	No	No

Dose-response curves for the biosensors were constructed after 12, 18 and 21 hours of growth in the presence of E2. These curves are located in appendix C. Figure 8 below contains the dose-response curve for 12 hours of growth. The dose-response curve is not fully resolved, so the growth time is not long enough. As the growth time is increased, the dose-response curve becomes more resolved. As the time is increased to 18 hours and greater, the dose-response curve becomes overgrown, and the sigmoidal curve becomes more difficult to resolve. The optimal growth time was determined to be 15 hours.

ERβ-H Dose-Response Curve – E2 - 34 C - 12 Hours - 8/22/11

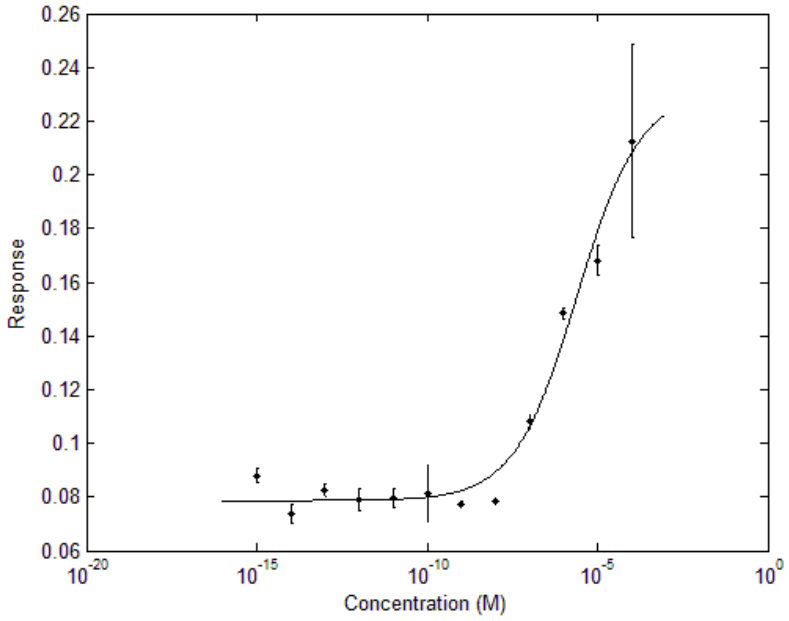

Figure 8: Dose-response curve for E2 for 12 hours growth

After the optimal growth time for the cells was determined, the cells were tested with weaker agonists. By testing with weaker agonists, the sensitivity of the assay could be initially tested. The compounds tested were E2, bisphenol A and *meso*-hexestrol. The dose-response curves for these tests are located in appendix C. The EC50 values for all three of these compounds are located in table 2 on the next page. E2 and *meso*-hexestrol have similar EC50

values. Bisphenol A did not have an EC50 value. These preliminary results showed that the assay was very sensitive to weaker agonists. Because the biosensors responded to not only strong estrogens but also weaker estrogens, the cells could be used to screen for estrogenic properties. The false negative result for bisphenol A was attributed to inadequacies in the experimental procedure, discussed above. The protocol changes, which were not adopted until after the initial testing, removed these false negatives. This result will be discussed in the screening results section.

Table 2: EC50 values for weaker estrogenic compounds

Compound	EC50 (M)
E2	$9.1*10^{-7}$
Bisphenol A	None
meso-hexestrol	$9.5*10^{-7}$

Liquid Handling Results

Calibration curves for the liquid handing robot are located in appendix D. The maximum concentration of dye had to be determined. If the dye concentration was too high, the plate reader cannot read the concentration accurately. A 20-fold dilution of the initial dye stock was determined to read within the limits of the plate reader. Hand pipetting established baseline for precision requirements for the robot. Figure 9 on the next page contains the hand pipetting curve for 20 to 200 microliters of dye solution. The graph displays a linear trend. Because the trend is linear, the amount of dye being transferred is considered precise.

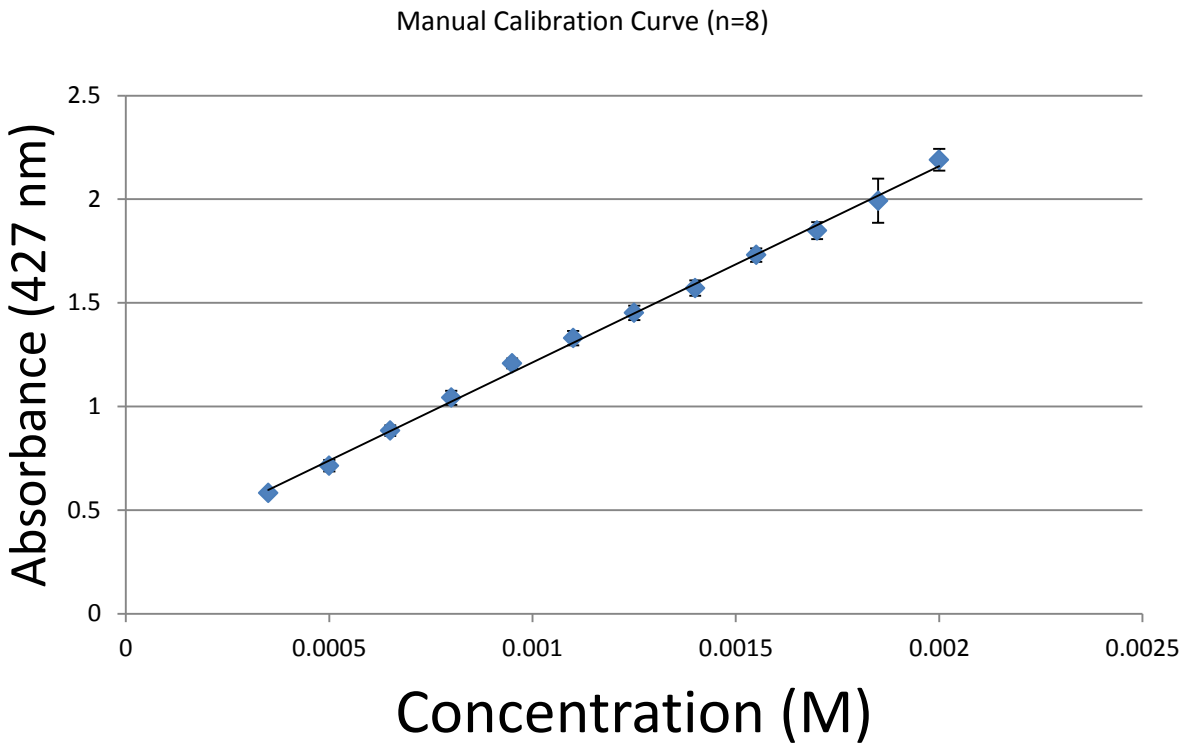

Figure 9: Liquid handling hand calibration curve

For each calibration, 8 replicates were made. This number was chosen because there are 8 rows on a single 96-well plate. Table 3 below summarizes the results of the calibration experiments. Each trial had a comparable slope. The standard deviations for each trial were also small. Finally, the R^2 value of each fit is high. The 1-20 microliter tool had a lower R^2 value than the other two calibrations. This is expected, because the concentration of dye in this calibration is very low. Because the calibrations show consistent and accurate data, the liquid handling robot can be accurately used to transfer liquids.

Table 3: Liquid handling calibration results summary

Experiment	Slope	R^2 value	Standard deviation (average)
Hand calibration	946.61	0.9979	0.038
20-200 microliter Tool	978.53	0.9995	0.025
1-20 microliter tool	997.76	0.9709	0.019

Absorbance Validation Results

The slopes of regressed lines from liquid handling calibration were used to verify the extinction coefficient of tartrazine dye. Tartrazine has an extinction coefficient of $2.5\pm0.2*10^4$ liters per mole centimeter [22]. By knowing the slope of the absorption curve and the path length of the plate reader, the extinction coefficient can be calculated. One issue with this method is that the plate reader used incorporates a feature called path length correction [23]. Path length correction scales the absorption value to account for the varying path lengths possible in 96-well plates and to account for other experimental factors. Therefore, instead of calculating the true path length of the samples and comparing to the actual plate geometry, a "corrected path length" can be calculated and compared. Table 4 below contains the calculated corrected path lengths for each calibration trial. Because the corrected path lengths are very similar, the plate reader can be used to accurately read cell growth on the media.

Table 4: Absorbance validation parameters

Experiment	Slope (absorbance/(mol/L)	Extinction coefficient (L/mol*cm)	Corrected path length (cm)
Hand calibration	946.61	$2.5*10^4$	0.038
20-200 microliter Tool	978.53	$2.5*10^4$	0.039
1-20 microliter tool	997.76	$2.5*10^4$	0.040

Screening Results

Thirty compounds were tested. These compounds came from the list in appendix A. Each compound was grown with three controls: E2, genestein and ICI182780. E2 is an estrogen agonist and a positive control. Genestein is a weak agonist and a positive control. ICI182780 is an estrogen antagonist and a negative control. To assure that growth was not plate-dependent, the controls were run on each plate. The dose-response curve of each compound was graphed with its corresponding controls. If the controls failed to work, the test was considered a failure.

One issue that quickly arose was the rejection of inaccurate dose-response curves. This inspection was done manually to assure that the curves rejected were indeed false matches. Figure 10 below contains a representative graph of this case. A dose-response curve was rejected if the data did not show a significant response or if the response was antagonistic. Appendix E contains all the dose-response curves for all thirty compounds. Appendix F contains the results summarized in a table.

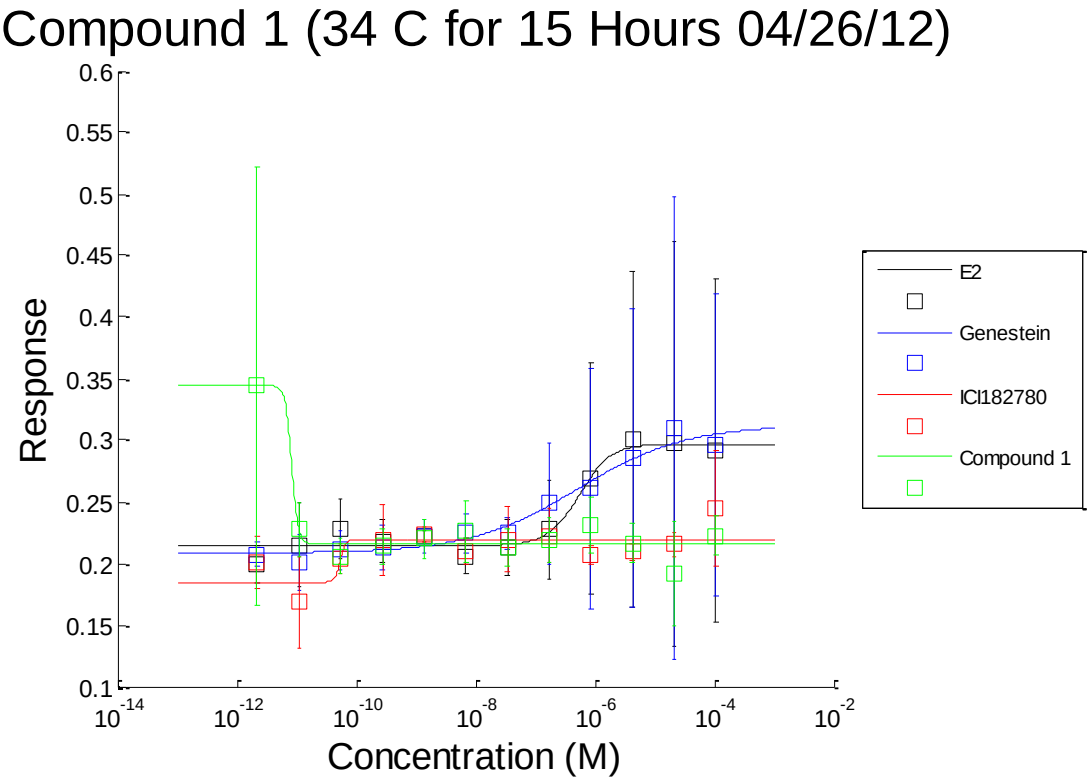

Figure 10: Representative dose-response curve with false match

Seven estrogen agonists were identified with the assay. There were 23 compounds that were identified as compounds with non-agonistic behavior. On all the trials, the controls performed as expected. E2 and genestein behaved as an agonist in all cases. ICI 182,780 behaved as a non-antagonist in all cases. Because the controls behaved as expected, the screening experiment performed as expected.

Comparison with Previous Work

Previous work involving the estrogen biosensor was completed using culture tubes, rather than 96-well plates, for growing bacteria [14]. The OD of the bacteria was measured, and dose-response curves were constructed from the data. Figure 11 below shows the dose-response curves for E2 and genestein. Both of these compounds have an EC50 in the concentration range of 0.1 micromoles per liter. These results agree with the results from trial controls.

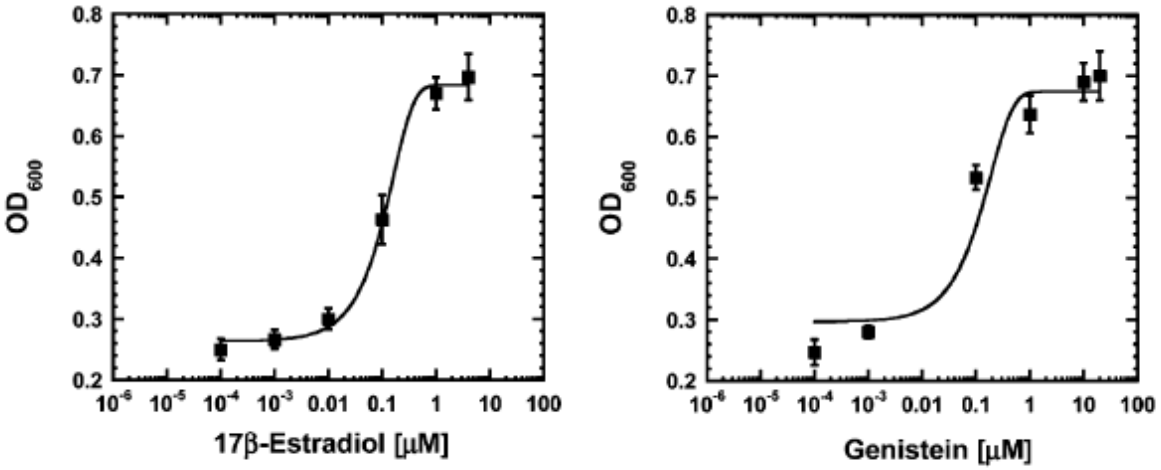

Figure 11: Dose-response curves for E2 and genestein [14]

Conclusions

The screening system performs well with controls. The system was able to identify estrogen agonists without the use of animal testing. In order to verify the results, however, animal models could be used to test the compounds which show an agonistic relationship. By eliminating out several compounds as estrogen agonists before using animal models, this system provides a preliminary estrogen screening method that provides several benefits to pharmaceutical and toxicological research.

In order for this screening system to be used in a high-throughput system, the experiment will have to be scaled to function with higher count well plates, such as 384- or 1596-well plates. The biological aspects of the experiment, such as growth media composition, will not be affected. An alternative liquid handling system will need to be adopted and validated. The mixing dynamics of 384- and 1596-well plates need to be investigated, before they are used in the system. Finally, a plate reader compatible with higher well count plates will need to be used. Other than the validation necessary for new equipment, the system remains essentially unchanged.

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Appendix A: ICCVAM Compounds

Table 5: ICCVAM compound list

Compound	Expected Behavior
17-α-ethinyl estradiol	Strong ER agonist
Diethylstilbestrol	Strong ER agonist
17α-estradiol	ER agonist
17β-estadiol	Strong ER agonist
Meso-hexestrol	Strong ER agonist
Zeralenone	ER agonist
Estrone	Strong ER agonist
Methyl testosterone	ER agonist
Coumestrol	ER agonist
Genistein	Weak ER agonist/antagonist
p-n-nonylphenol	ER agonist/antagonist
Bisphenol B	ER agonist
Diadzein	Weak ER agonist
4-cumylphenol	Weak ER agonist
Bisphenol A	ER agonist
o,p'-DDT	Weak ER agonist/antagonist
p,p'-methoxychlor	Weak ER agonist
Fenarimol	---
Apigenen	ER agonist
Tamoxifen	ER antagonist
Kepone	Binds to ER
Butylbenzyl phthalate	ER agonist
4-hydroxytamoxifen	ER antagonist
Kaempferol	ER agonist
4-tert-octylphenol	ER agonist
p,p'-DDE	---
Di-*n*-butyl phthalate	ER agonist
Flavone	Weak ER antagonist
Dexamethasone	---
5α-dihydro-testosterone	Weak ER agonist
2,4,5-trichloro-phenoxyacetic acid	Weak ER agonist
Dibenzo[*a,h*]-anthracene	---
ICI 182,780	ER antagonist
Atrazine	---
Progesterone	---
Testosterone	---
Corticosterone	---
Phenobarbital	---
Vinclozolin	---
Cyproterone acetate	---
Flutamide	---

Linuron	---
Methyltrienolone	---
Mifepristone	---
Procymidone	---
Clomphene citrate	Binds to ER, selective estrogen receptor modulator
Ethyl paraben	Binds weakly to ER
Norethynodrel	Binds to ER
Actinomycon D	---
4-androstenedione	---
2-*sec*-butylphenol	---
Diethylhexyl phthalate	Does not bind ER
fadrozole	---
Fluoroanthene	---
Hydroxyflutamide	---
Morin	Binds weakly to ER
Phenophtalin	---
Propylthiouracil	--
Sodium azide	---
12-*o*-tetradecanoyl-phorbol-13-acetate	---
Ammonium perchlorate	---
Anastrole	---
Apomorphine	---
Bicalutamide	---
CGS 18320B	---
Cycloheximide	---
Finasteride	---
Fluoxymestrone	---
Haloperidol	---
Ketoconazole	---
Medroxyprogesterone acetate	---
Nilutamide	---
Oxazepam	---
Pimozide	---
Reserpine	---
Spironolactone	---
L-thyroxine	---
17β-trenbolone	---

Appendix B: Media Recipes

LB Agar Plates (1L)
~Add the following to 1L Ultrapure H_2O:
1.5% Agar → 15g
1% Tryptone → 10g
1% NaCl – 10g
0.5% Yeast Extract – 5g
~Adjust pH to 7.0
~Autoclave solution
~Let cool until touchable (think hot coffee)
~Add the following:
50 µg/mL Thymine – 25ml 2mg/mL Thymine
200 µg/mL AMP → 8ml AMP_{25}
~Dispense into plates and let cool until firm
~Store plates at 4ºC

AMP_{25} – 25mg/mL Ampicillin (50mL)
~Dissolve in diH_2O
~Sterilize by filtration
~Store at -20ºC

2mg/mL Thymine (200mL)
~Dissolve in diH_2O
~Sterilize by autoclaving
~Store at room temperature

LB (1L)
~Add the following to 1L Ultrapure H_2O
1% Tryptone → 10g
1% NaCl → 10g
0.5% Yeast Extract → 5g
~Adjust pH to 7.0
~Sterilize by autoclaving
~Store at room temperature

0.1 M $CaCl_2$ (200mL)
~Dissolve 2.94g in 200mL diH_2O
~Sterilize by autoclaving
~Store at 4ºC

Minimal Davis Broth (1L)
~Add the following to 1L diH_2O:
Dipotassium phosphate – 35g
Monopotassium phosphate – 10g
Sodium citrate – 2.5g
Magnesium Sulfate – 0.5g
Ammonium Sulfate – 5g
~Sterilize by autoclaving
~Store at 4ºC

10% Casamino Acid (200mL)
~Dissolve 20g Casamino Acid into 200mL diH_2O
~Sterilize by vacuum filtration
~Store at 4ºC

20% Glucose (200mL)
~Dissolve 40g Dextrose into 200mL diH_2O
~Sterilize by vacuum filtration
~Store at 4ºC

1% Thiamine HCl (100mL)
~Dissolve 1g Thiamine HCl into 100mL diH_2O
~Sterilize by vacuum filtration
~Store at 4ºC

Thy Pool (200mL)
~Dissolve the following in 200mL diH_2O:
L-Arg – 400mg
L-His – 400mg
L-Leu – 400mg
L-Meth – 400mg
L-Pro – 400mg
L-Thr – 400mg
~Sterilize by vacuum filtration
~Store at 4ºC

-Thy (1L)
~Add the following to 761mL Ultrapure H_2O:
20% Glucose – 10ml
Thy Pool – 10ml
10% Casamino Acid – 10ml
1% Thiamine HCl - 200µl
0.1M $CaCl_2$ – 1ml
Minimum Davis Broth – 200ml
200 µg/mL AMP → 8ml AMP_{25}
~Store at 4ºC

Appendix B: Curve-Fitting Programs

```
% ICCVAM Data Processing Program
% This program reads an Excel .xls file with multiple sheets
% And Creates Dose-response curves

% Written for n=3
% Load data from sheet
sheet1=xlsread('01102012.xls',1);
sheet2=xlsread('01102012.xls',2);
sheet3=xlsread('01102012.xls',3);

% Name controls, compounds in plate
controls={'E2', 'Genestein', 'ICI182780'};
compounds={'compound1', 'compound2', 'compound3', 'compound4', 'compound5'};

% Make arrays for each compound
% Compound 1
compound1_1=sheet1(2,:);
compound1_2=sheet2(2,:);
compound1_3=sheet3(2,:);
% Compound 2
compound2_1=sheet1(3,:);
compound2_2=sheet2(3,:);
compound2_3=sheet3(3,:);
% Compound 3
compound3_1=sheet1(4,:);
compound3_2=sheet2(4,:);
compound3_3=sheet3(4,:);
% Compound 4
compound4_1=sheet1(5,:);
compound4_2=sheet2(5,:);
compound4_3=sheet3(5,:);
% Compound 5
compound5_1=sheet1(6,:);
compound5_2=sheet2(6,:);
compound5_3=sheet3(6,:);
% Compound 6
compound6_1=sheet1(7,:);
compound6_2=sheet2(7,:);
compound6_3=sheet3(7,:);
% Compound 7
compound7_1=sheet1(8,:);
compound7_2=sheet2(8,:);
compound7_3=sheet3(8,:);
```

```
% Compound 8
compound8_1=sheet1(9,:);
compound8_2=sheet2(9,:);
compound8_3=sheet3(9,:);

% Write to files
concentration=[1e-4; 2e-5; 4e-6; 8e-7; 1.6e-7; 3.2e-8; 6.4e-9; 1.28e-9; 2.56e-10; 5.12e-11;
1.024e-11; 2.048e-12];
% Write file 1
xlswrite('compound1.xls',concentration,'a1:a12')
xlswrite('compound1.xls',rot90(compound1_1,3),'b1:b12')
xlswrite('compound1.xls',rot90(compound1_2,3),'c1:c12')
xlswrite('compound1.xls',rot90(compound1_3,3),'d1:d12')
% Write file 2
xlswrite('compound2.xls',concentration,'a1:a12')
xlswrite('compound2.xls',rot90(compound2_1,3),'b1:b12')
xlswrite('compound2.xls',rot90(compound2_2,3),'c1:c12')
xlswrite('compound2.xls',rot90(compound2_3,3),'d1:d12')
% Write file 3
xlswrite('compound3.xls',concentration,'a1:a12')
xlswrite('compound3.xls',rot90(compound3_1,3),'b1:b12')
xlswrite('compound3.xls',rot90(compound3_2,3),'c1:c12')
xlswrite('compound3.xls',rot90(compound3_3,3),'d1:d12')
% Write file 4
xlswrite('compound4.xls',concentration,'a1:a12')
xlswrite('compound4.xls',rot90(compound4_1,3),'b1:b12')
xlswrite('compound4.xls',rot90(compound4_2,3),'c1:c12')
xlswrite('compound4.xls',rot90(compound4_3,3),'d1:d12')
% Write file 5
xlswrite('compound5.xls',concentration,'a1:a12')
xlswrite('compound5.xls',rot90(compound5_1,3),'b1:b12')
xlswrite('compound5.xls',rot90(compound5_2,3),'c1:c12')
xlswrite('compound5.xls',rot90(compound5_3,3),'d1:d12')
% Write file 6
xlswrite('compound6.xls',concentration,'a1:a12')
xlswrite('compound6.xls',rot90(compound6_1,3),'b1:b12')
xlswrite('compound6.xls',rot90(compound6_2,3),'c1:c12')
xlswrite('compound6.xls',rot90(compound6_3,3),'d1:d12')
% Write file 7
xlswrite('compound7.xls',concentration,'a1:a12')
xlswrite('compound7.xls',rot90(compound7_1,3),'b1:b12')
xlswrite('compound7.xls',rot90(compound7_2,3),'c1:c12')
xlswrite('compound7.xls',rot90(compound7_3,3),'d1:d12')
% Write file 8
xlswrite('compound8.xls',concentration,'a1:a12')
xlswrite('compound8.xls',rot90(compound8_1,3),'b1:b12')
```

```
xlswrite('compound8.xls',rot90(compound8_2,3),'c1:c12')
xlswrite('compound8.xls',rot90(compound8_3,3),'d1:d12')

% Graph files
% Need associated sigmoid processing files ec50, ec50find, hillcal,
% init_coeffs and sigmoid
% Creates five graphs

% Graph each compound
for n=1:1:5
   figure;
   hold on;
   ec50find('compound6.xls','k','ks')
   ec50find('compound7.xls','b','bs')
   ec50find('compound8.xls','r','rs')
   ec50find(strcat('compound',int2str(n),'.xls'),'g','gs')
   leg=legend(controls{1},'',controls{2},'',controls{3},'',compounds{n},'')
   set(leg,'FontSize',15)
   titlevar=strcat(compounds{n},' Growth-Concentration Curve at 34 C for 19 Hours 11/11/11');
   title(titlevar, 'FontSize',20);
end
```

```
function EC50=EC50Find(file,a,b)
%EC50Find takes a string input, the name of the excel file where the data
%is stored, and returns the EC50 value and a plot of the data with the
%appropriate sigmoid fit.

if ~ischar(file)
   error('The input file name must be a string');
end

data=xlsread(file);

concentrations=data(:,1);
responses=data(:,2:end);
MeanResponse=mean(responses,2);
EBar=std(responses,0,2);

con=log10(concentrations);
results=ec50(con,MeanResponse);
r=results;

Min=min(concentrations);
Max=max(concentrations);
lowlim=floor(log10(Min))-1;
highlim=ceil(log10(Max))+1;
x=logspace(lowlim,highlim,1000);
X=log10(x);

y=r(1)+(r(2)-r(1))./(1+10.^((r(3)-X)*r(4)));

% a='b';
    m=plot(x,y,a);
   hold all
   n=errorbar(concentrations,MeanResponse,EBar,b);
   h=gca;
   set(h,'XScale','log')
%    legend('Fit','Data','Location','SouthEast')
   xlab=xlabel('Concentration (M)')
   ylab=ylabel('Response')
   set(xlab,'FontSize',15)
   set(ylab,'FontSize',15)
%    hold off

r(4)
EC50=10^r(3)
```

```
function results=ec50(conc,responses)
% EC50 Function to fit a dose-response data to a 4 parameter dose-response
%   curve.
%
% Requirements: nlinfit function in the Statistics Toolbox
%          and accompanying m.files: init_coeffs.m and sigmoid.m
% Inputs: 1. a 1 dimensional array of drug concentrations
%         2. the corresponding m x n array of responses
% Algorithm: generate a set of initial coefficients including the Hill
%              coefficient
%           fit the data to the 4 parameter dose-response curve using
%              nonlinear least squares
% Output: a matrix of the 4 parameters
%         results[m,1]=min
%         results[m,2]=max
%         results[m,3]=ec50
%         results[m,4]=Hill coefficient
%
% Copyright 2004 Carlos Evangelista
% send comments to CCEvangelista@aol.com
% Version 1.0    01/07/2004

[m,n]=size(responses);
results=zeros(n,4);
for i=1:n
    response=responses(:,i);
    initial_params=init_coeffs(conc,response);
    [coeffs,r,J]=nlinfit(conc,response,'sigmoid',initial_params);
%    disp (coeffs);
    for j=1:4
       results(i,j)=coeffs(j);
    end
end
%disp (results);
```

```
function yhat=sigmoid(params4,x)
% SIGMOID Function to fit data to a four parameter dose response curve
% requires the nlinfit function of the statistics toolbox and a set of
% initial parameters such as the one generated by init_coeffs.m.
% This function is used by ec50.m
%
% Copyright 2004 Carlos Evangelista
% send comments to CCEvangelista@aol.com
% Version 1.0    01/07/2004

min=params4(1);
max=params4(2);
ec=params4(3);
hillc=params4(4);

x1=x(:,1);

yhat=min+(max-min)./(1+10.^((ec-x1)*hillc));


function [concentrations,MeanResponse,EBar,y,x,X]= hillcal(file)

if ~ischar(file)
   error('The input file name must be a string');
end

 data=xlsread(file);

concentrations=data(:,1);
responses=data(:,2:end);
MeanResponse=mean(responses,2);
EBar=std(responses,0,2);

con=log10(concentrations);
results=ec50(con,MeanResponse);
r=results;

Min=min(concentrations);
Max=max(concentrations);
lowlim=floor(log10(Min))-1;
highlim=ceil(log10(Max))+1;
x=logspace(lowlim,highlim,1000);
X=log10(x);

y=r(1)+(r(2)-r(1))./(1+10.^((r(3)-X)*r(4)));
```

```
function init_params = init_coeffs(x,y)
% INIT_COEFFS Function to generate the initial parameters for the 4
% parameter dose response curve.
% Requires an array of doses and an array of responses
% This function is used by sigmoid.m and ec50.m
%
% Copyright 2004 Carlos Evangelista
% send comments to CCEvangelista@aol.com
% Version 1.0    01/07/2004

parms=ones(1,4);
parms(1)=min(y);
parms(2)=max(y);
parms(3)=(min(x)+max(x))/2;
sizey=size(y);
sizex=size(x);
if (y(1)-y(sizey))./(x(2)-x(sizex))>0
   parms(4)=(y(1)-y(sizey))./(x(2)-x(sizex));
else
   parms(4)=1;
end
init_params=parms;
```

Appendix C: Dose-Response Curves for Initial Validation

Biological Validation

ERβ-H Dose-Response Curve – E2 - 34 C - 18 Hours - 8/22/11

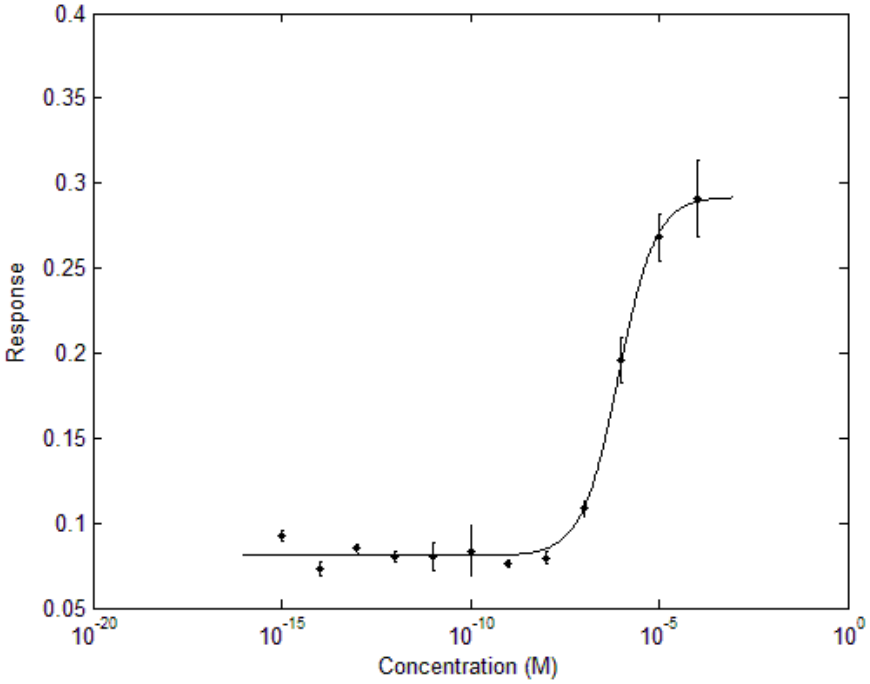

Figure 12: Initial dose-response curve for E2

ERβ-H Dose-Response Curve - T3 - 34 C - 18 Hours - 8/22/11

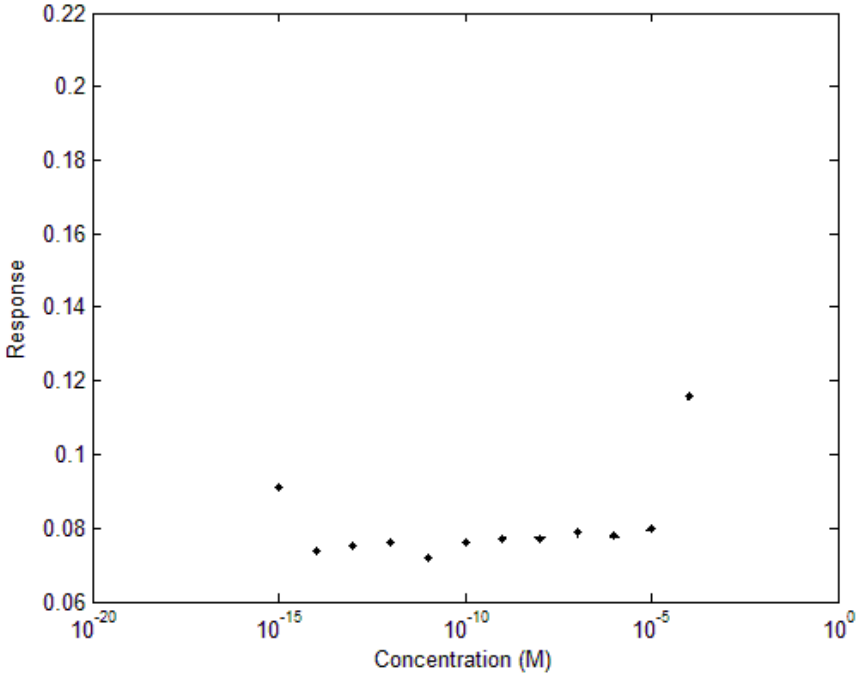

Figure 13: Initial dose-response curve for T3

ERβ-H Dose-Response Curve – Triac - 34 C - 18 Hours - 8/22/11

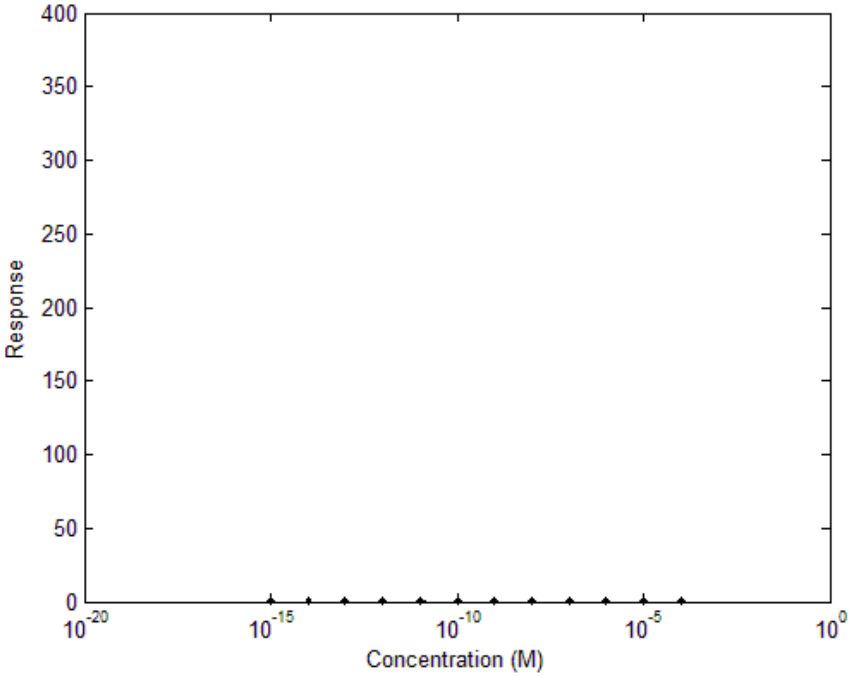

Figure 14: Initial dose-response curve for Triac

Growth Time Determination

ERβ-H Dose-Response Curve – E2 - 34 C - 12 Hours - 8/22/11

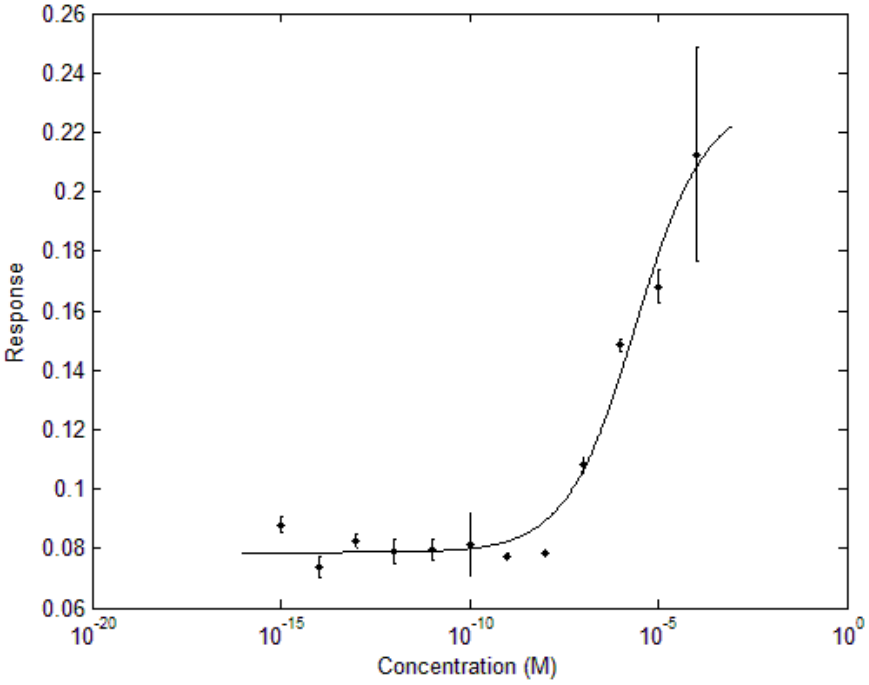

Figure 15: Growth time determination curve for 12 hours

ERβ-H Dose-Response Curve – E2 - 34 C - 18 Hours - 8/22/11

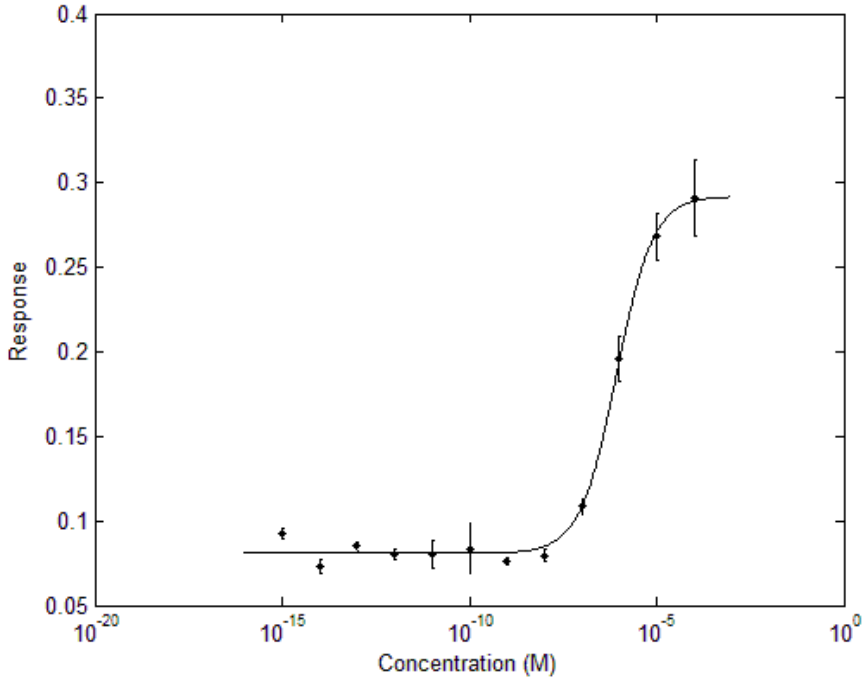

Figure 16: Growth time determination curve for 18 hours

ERβ-H Dose-Response Curve – E2 - 34 C - 21 Hours - 8/22/11

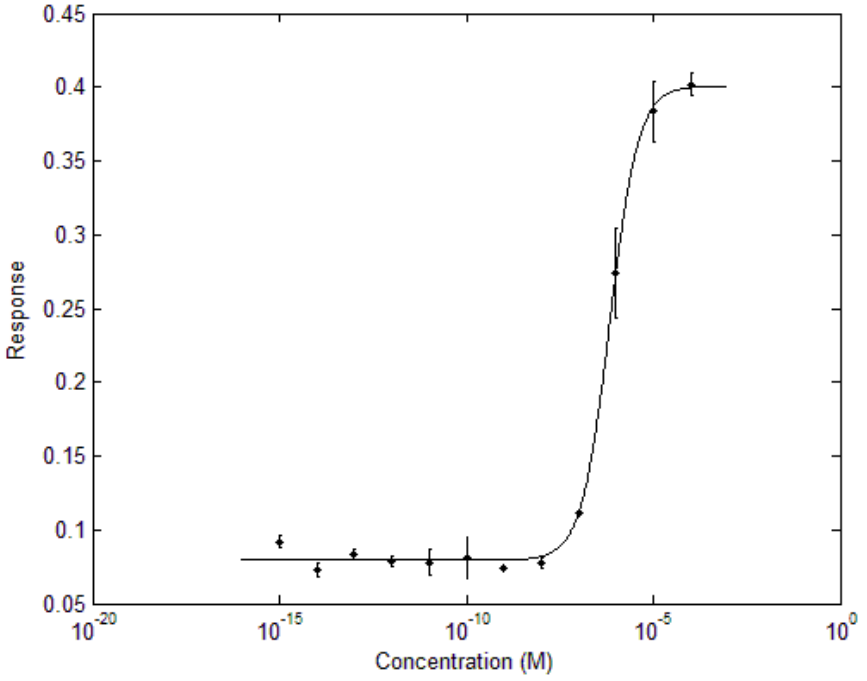

Figure 17: Growth time determination curve for 21 hours

Estrogen Sensitivity Test

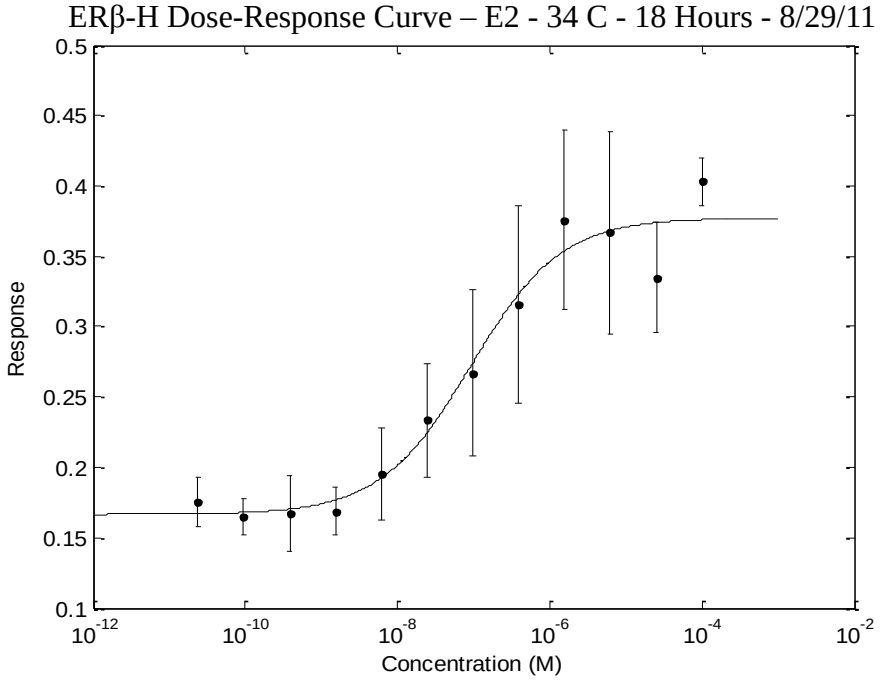

Figure 18: Estrogen sensitivity test for E2

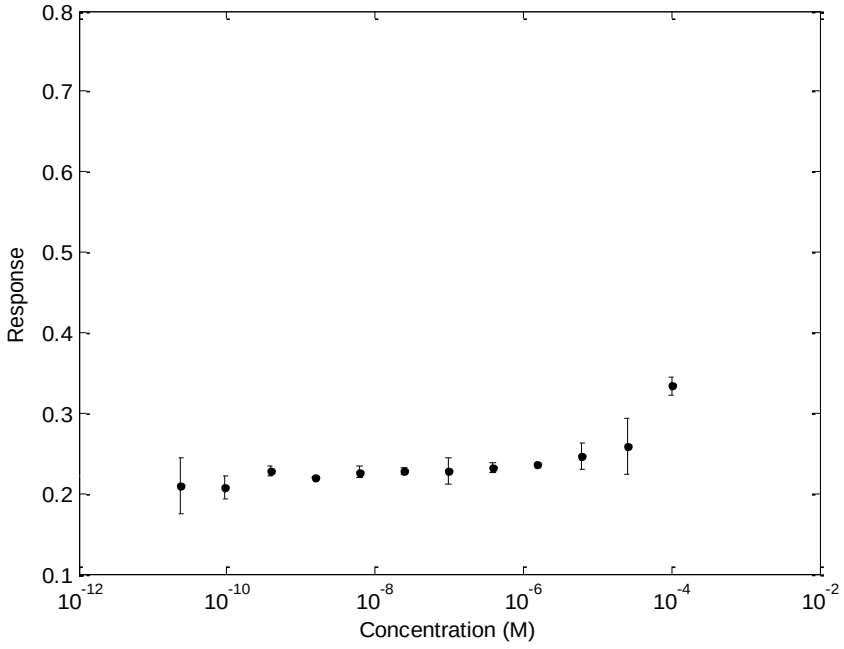

Figure 19: Estrogen sensitivity test for bisphenol A

ERβ-H Dose-Response Curve – *meso*-hexestrol - 34 C - 18 Hours - 8/29/11

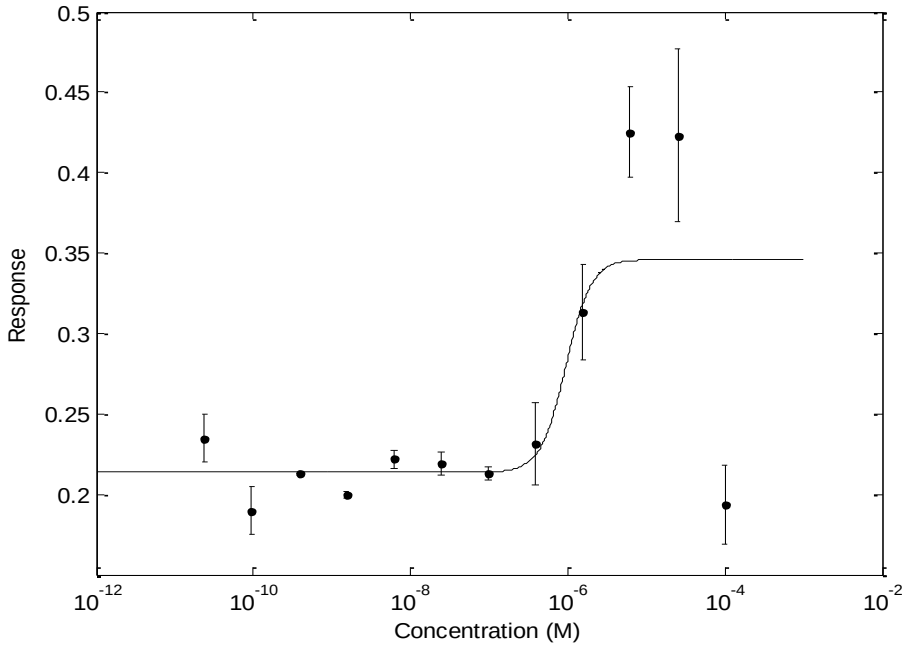

Figure 20: Estrogen sensitivity test for meso-hexestrol

Appendix D: Liquid Handling Calibration Graphs

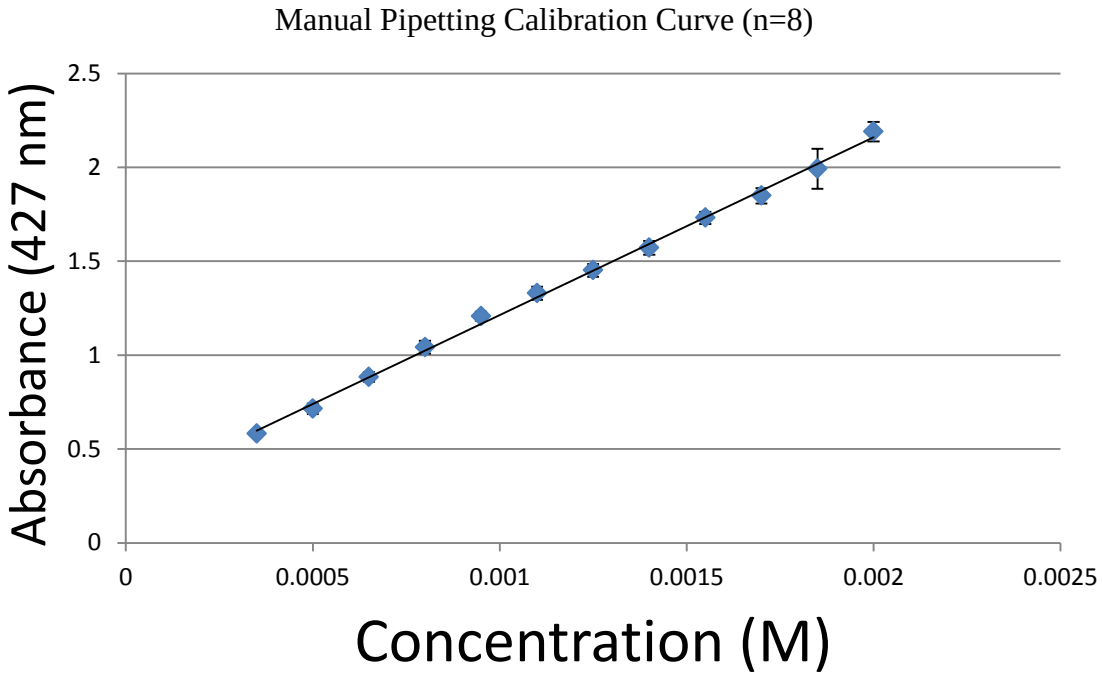

Figure 21: Manual calibration curve

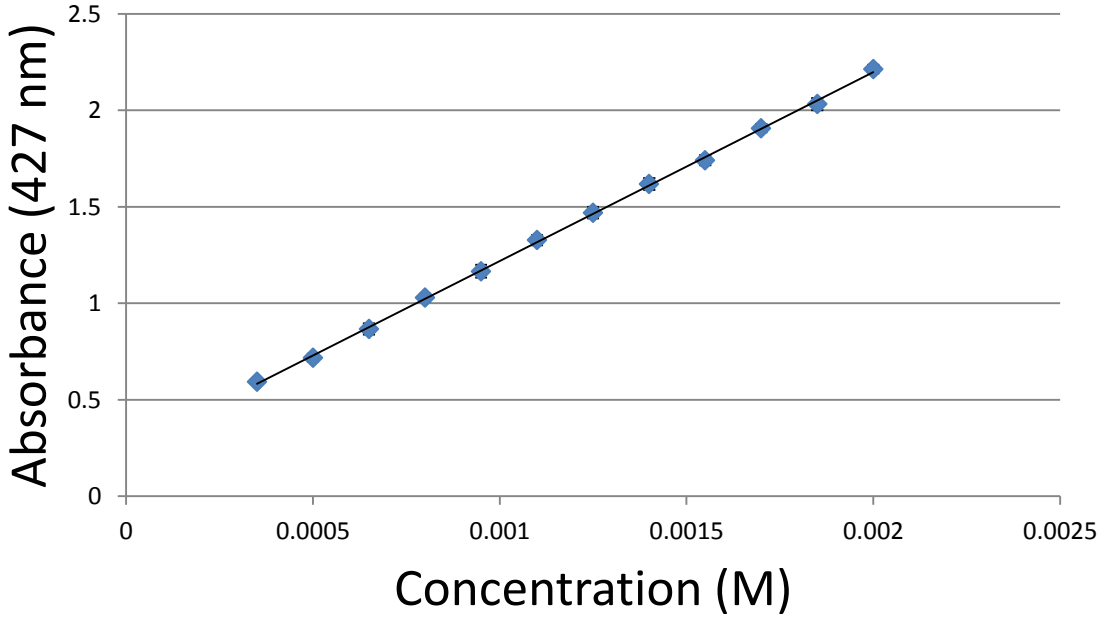

Figure 22: Robot calibration curve

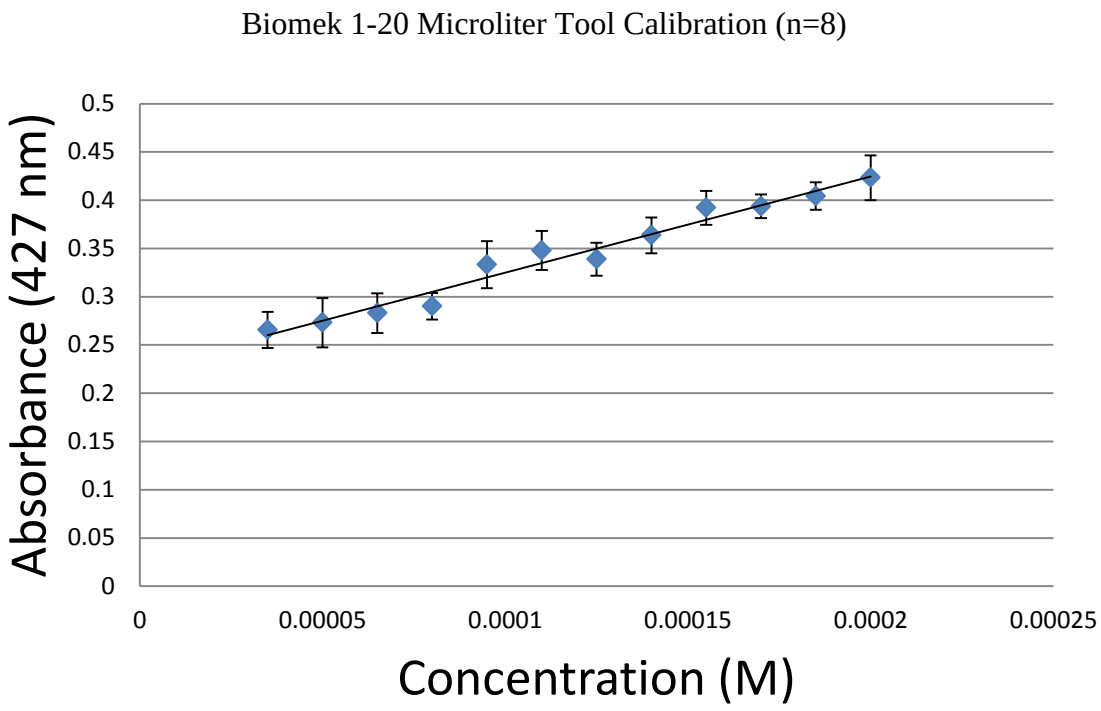

Figure 23: Robot calibration curve for lower concentrations

Appendix E: Full Screening Dose-Response Curves

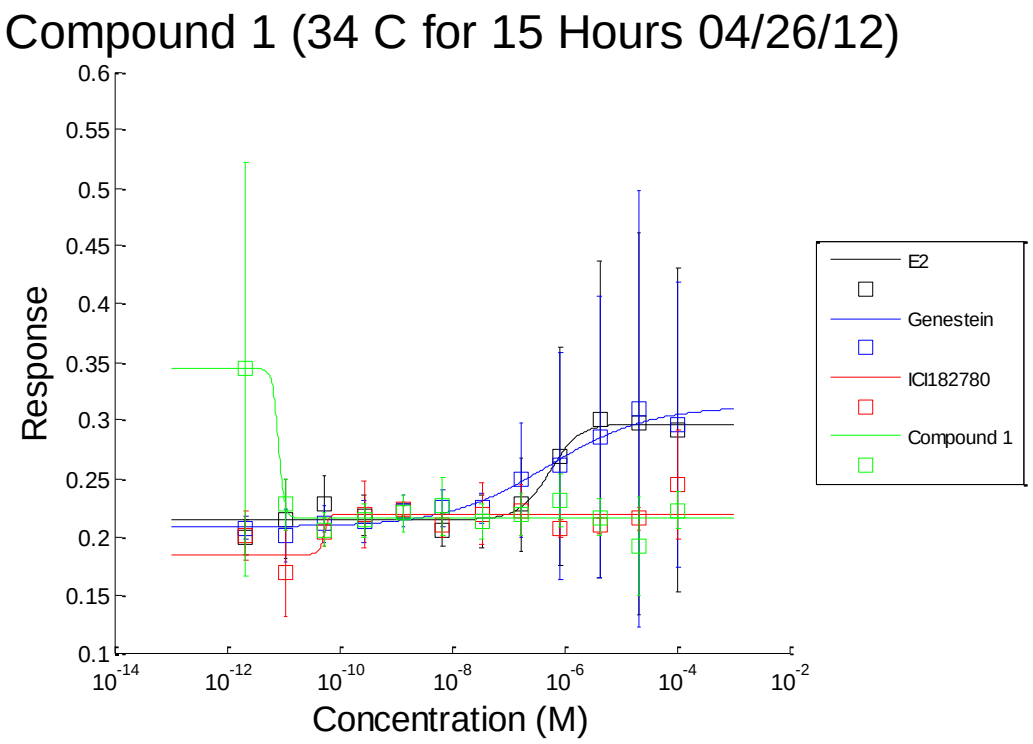

Figure 24: Dose-response curve for compound 1

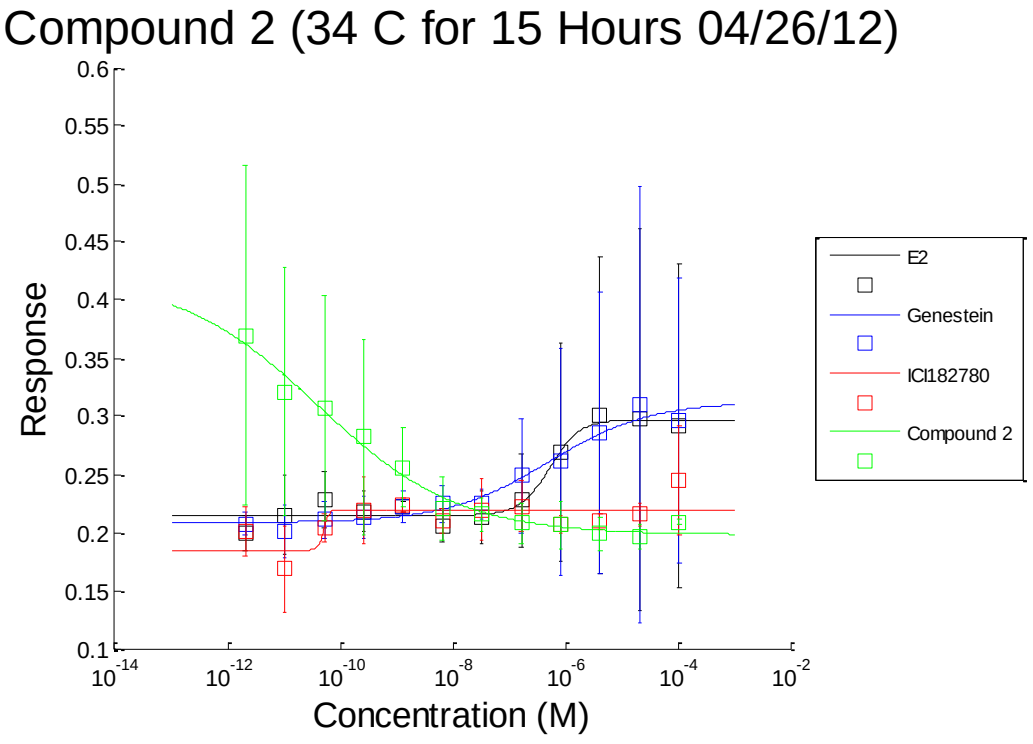

Figure 25: Dose-response curve for compound 2

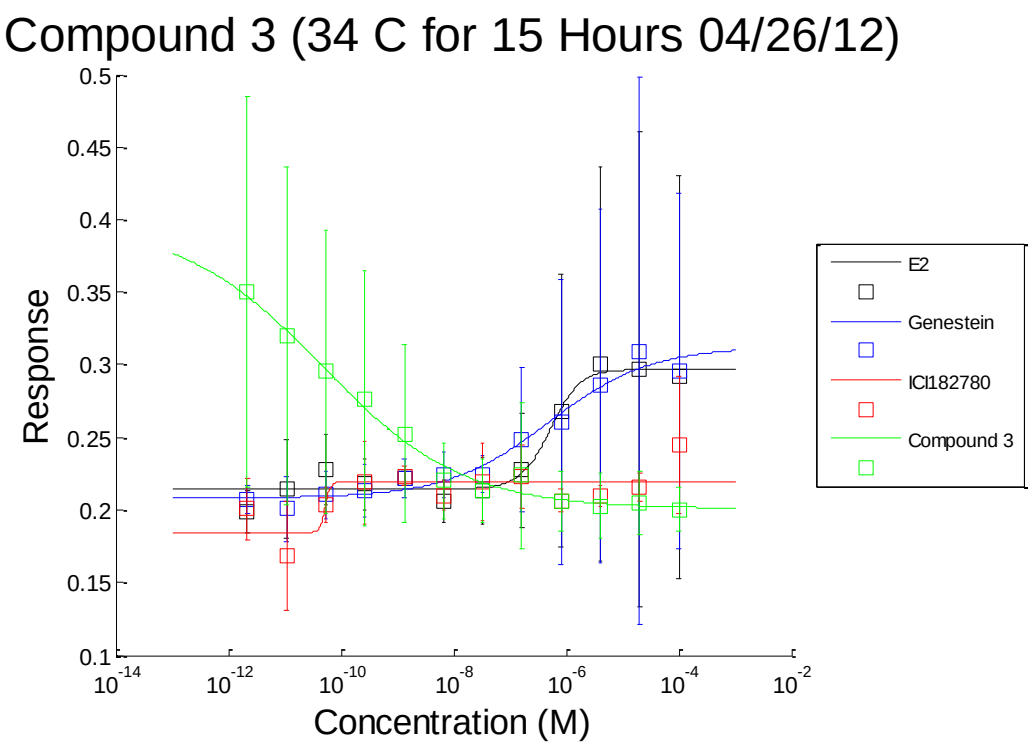

Figure 26: Dose-response curve for compound 3

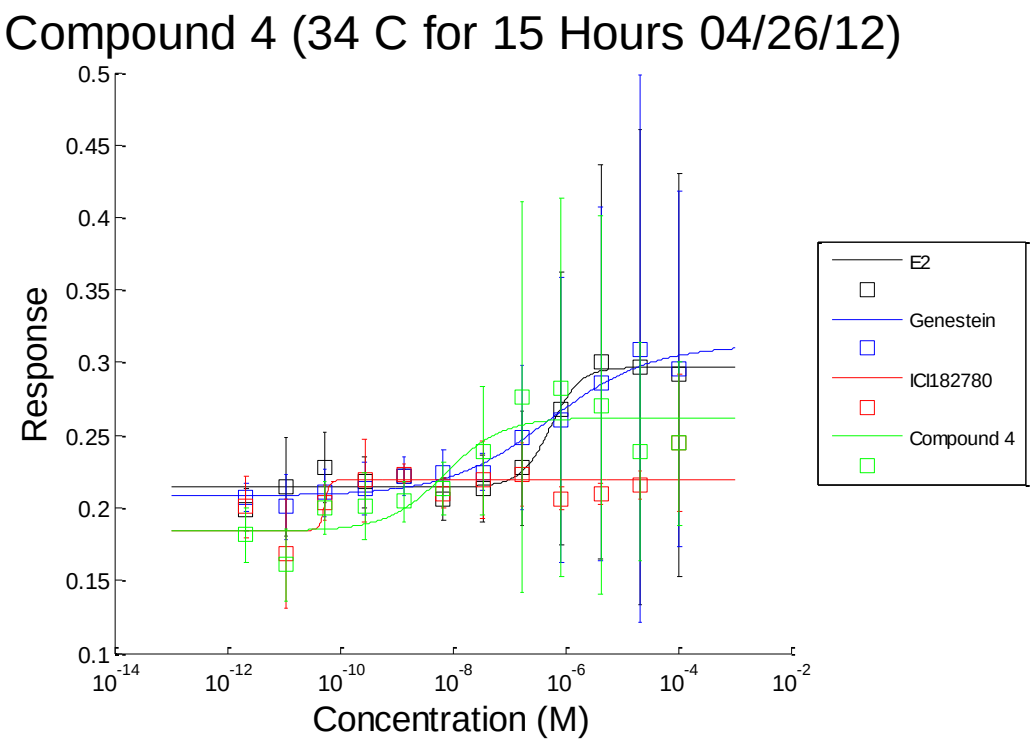

Figure 27: Dose-response curve for compound 4

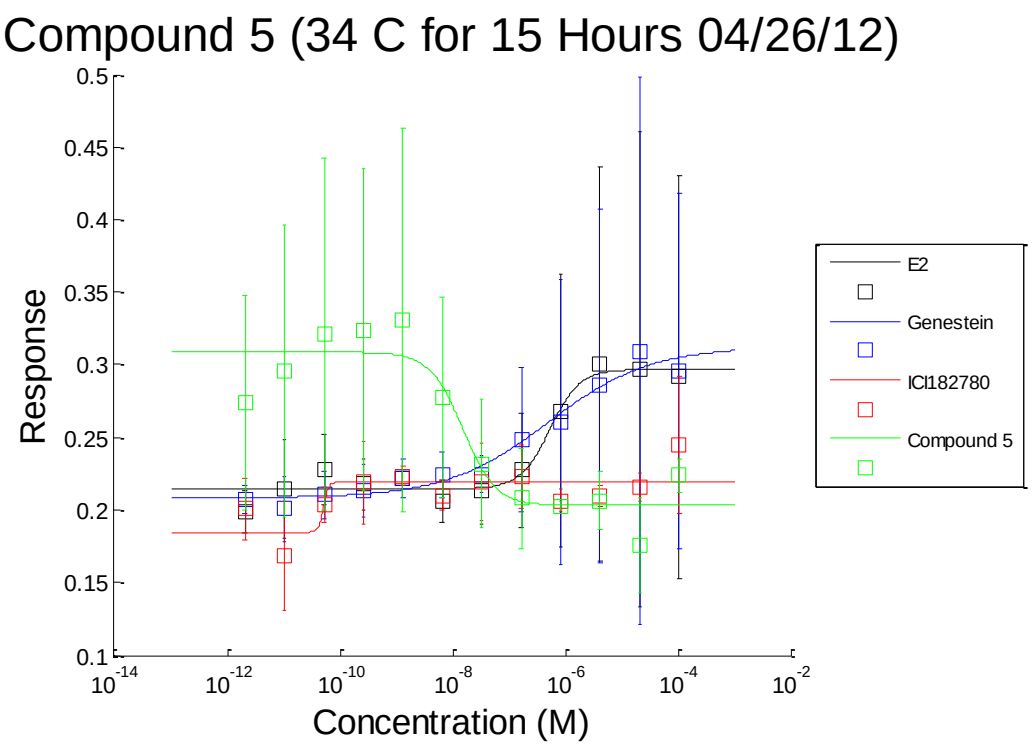

Figure 28: Dose-response curve for compound 5

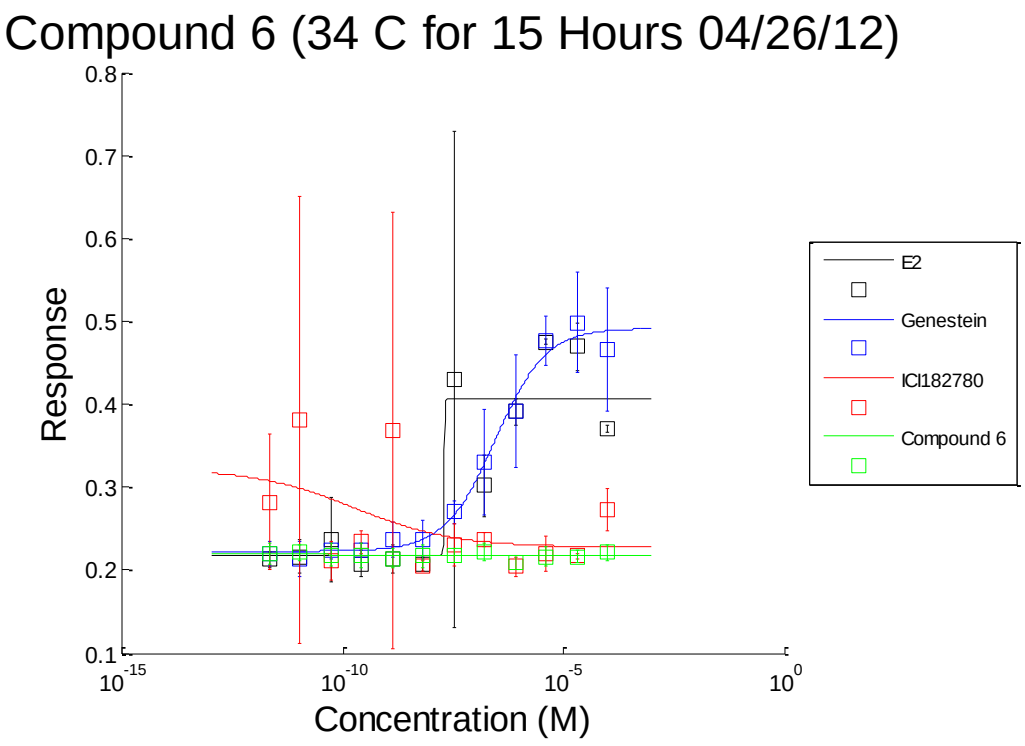

Figure 29: Dose-response curve for compound 6

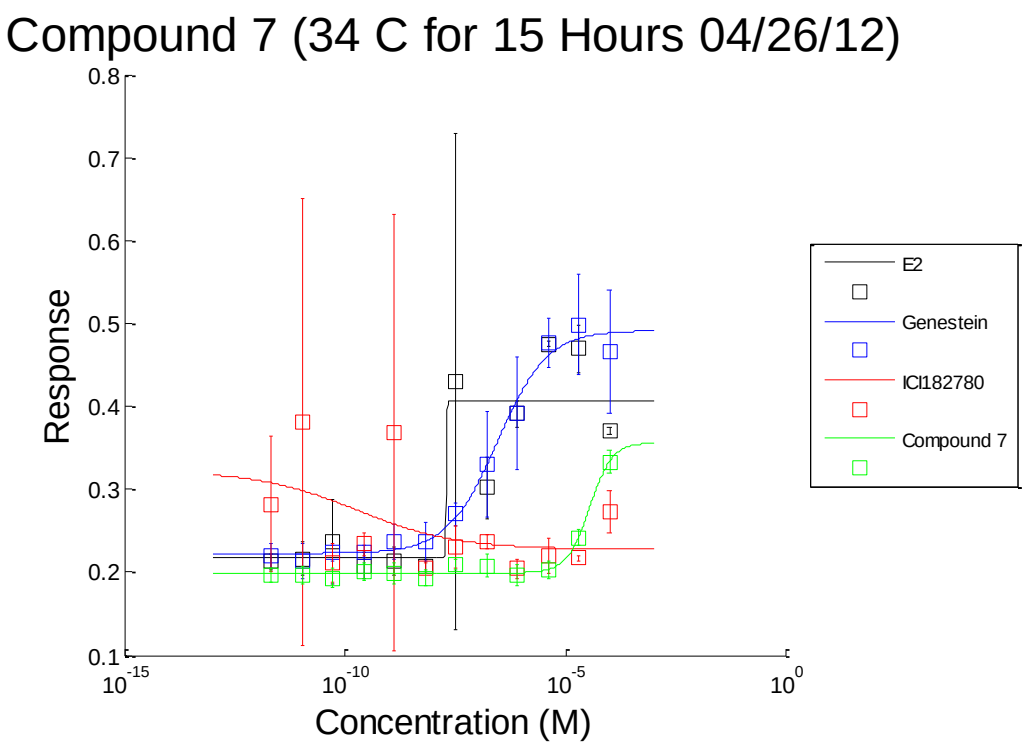

Figure 30: Dose-response curve for compound 7

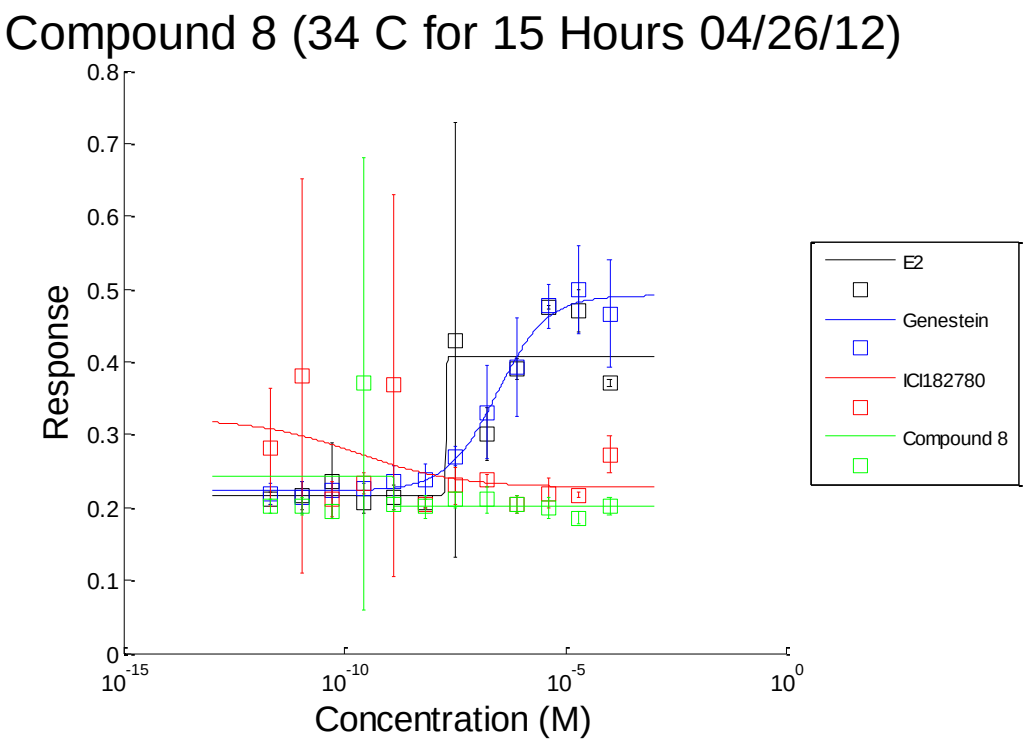

Figure 31: Dose-response curve for compound 8

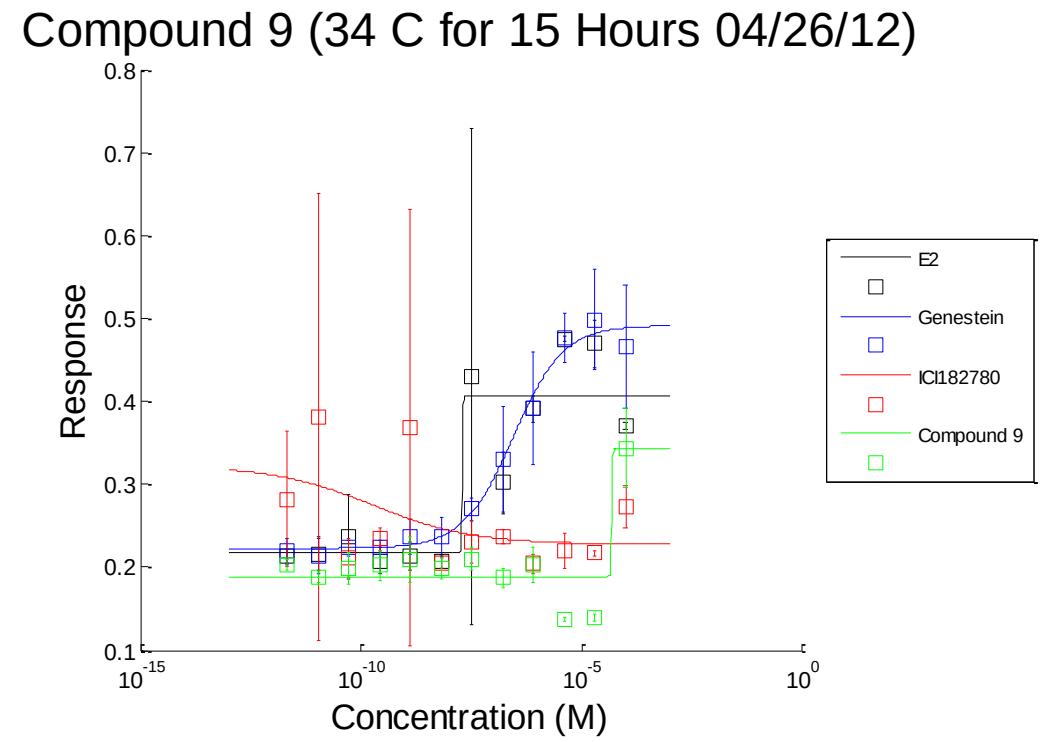

Figure 32: Dose-response curve for compound 9

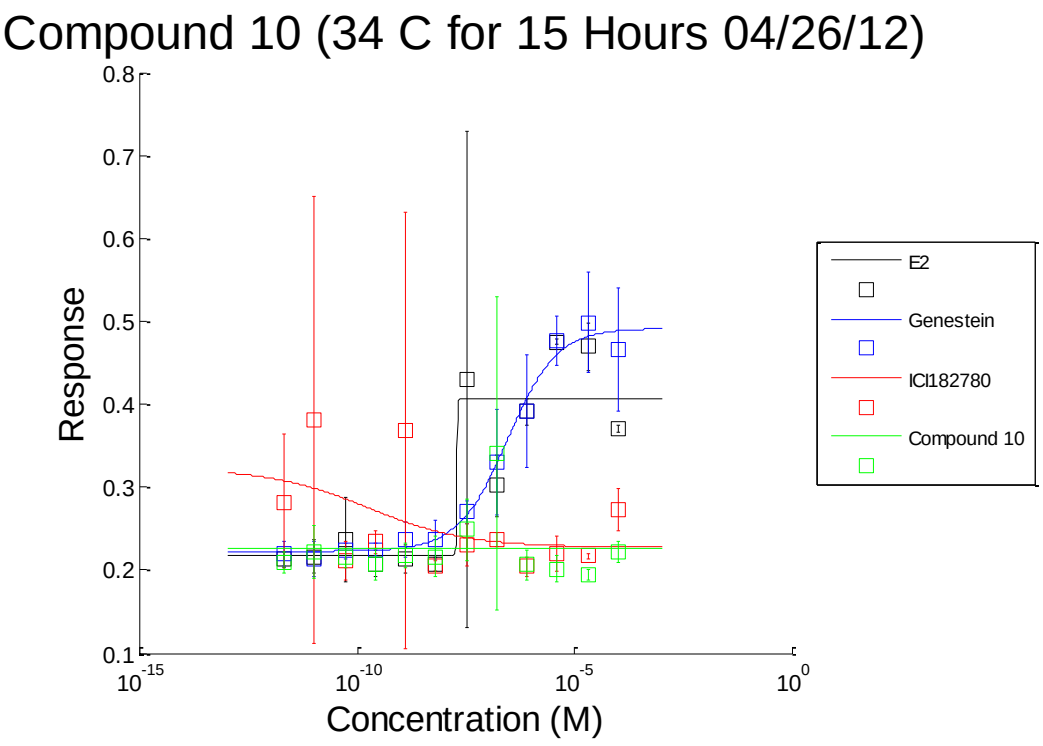

Figure 33: Dose-response curve for compound 10

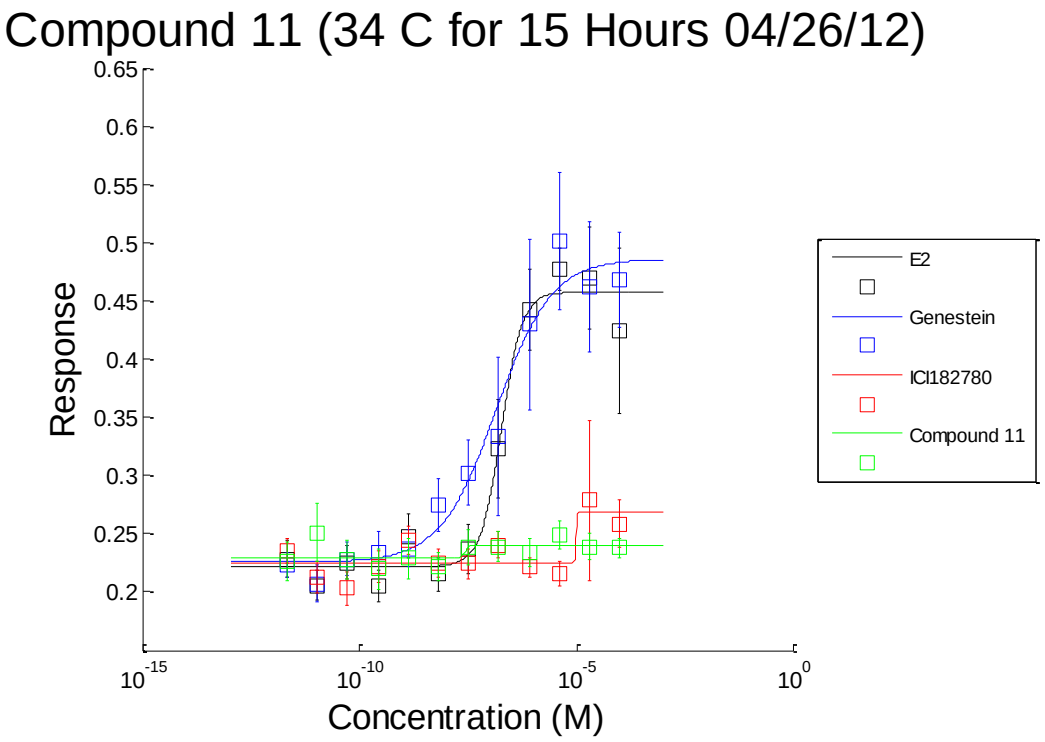

Figure 34: Dose-response curve for compound 11

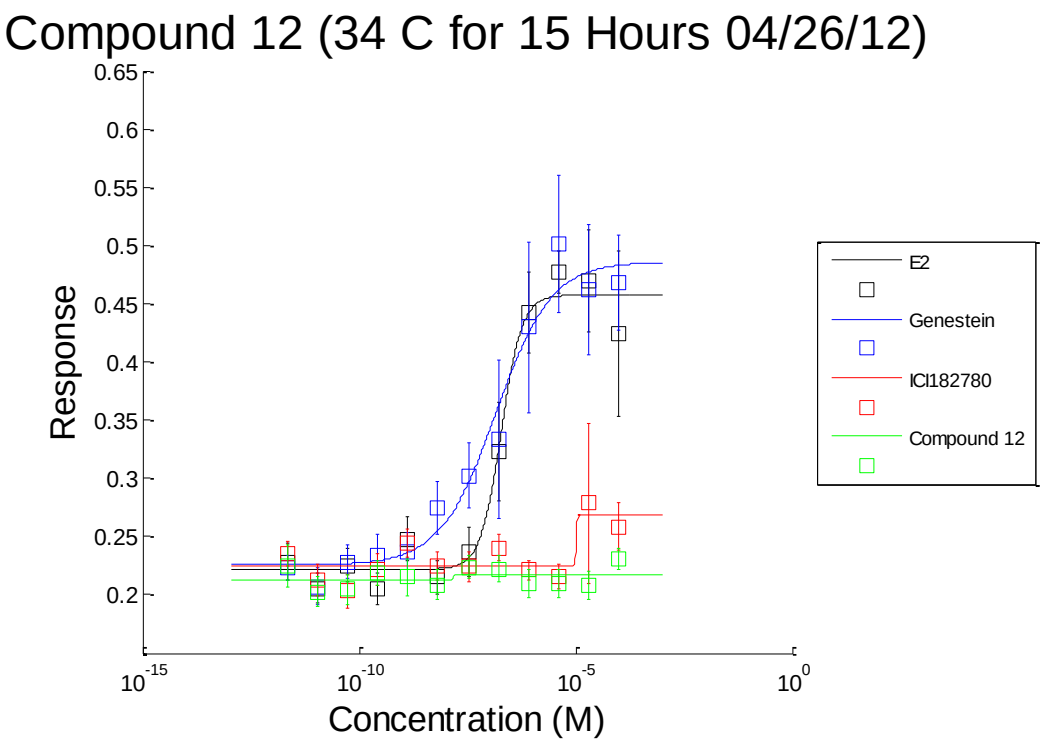

Figure 35: Dose-response curve for compound 12

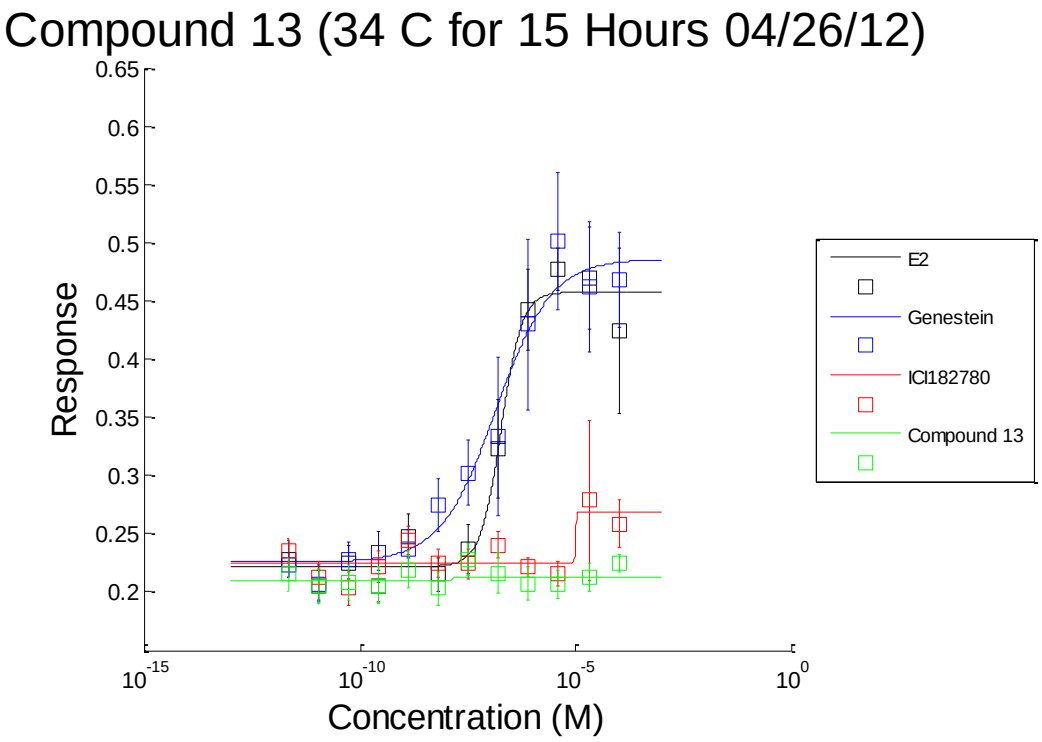

Figure 36: Dose-response curve for compound 13

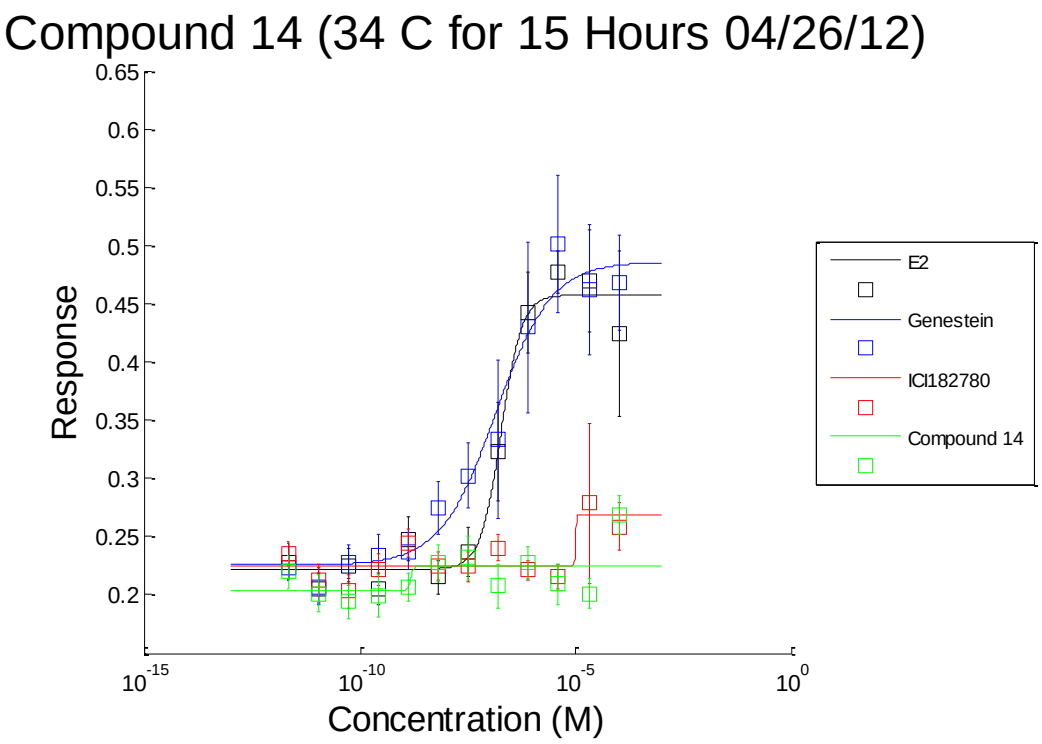

Figure 37: Dose-response curve for compound 14

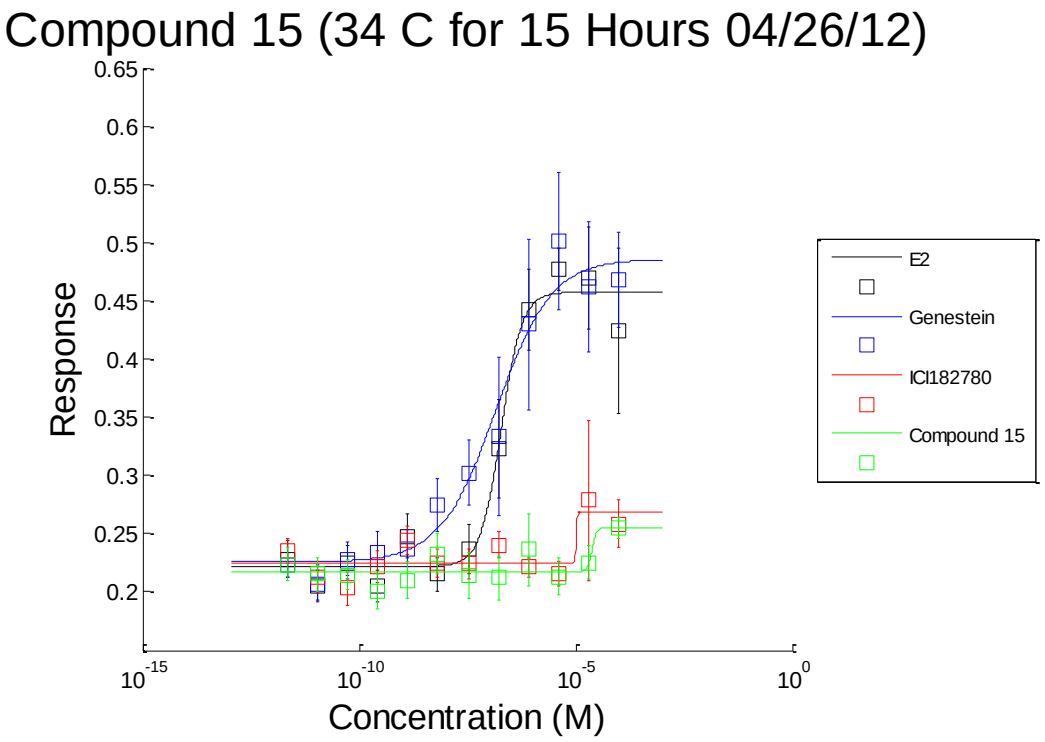

Figure 38: Dose-response curve for compound 15

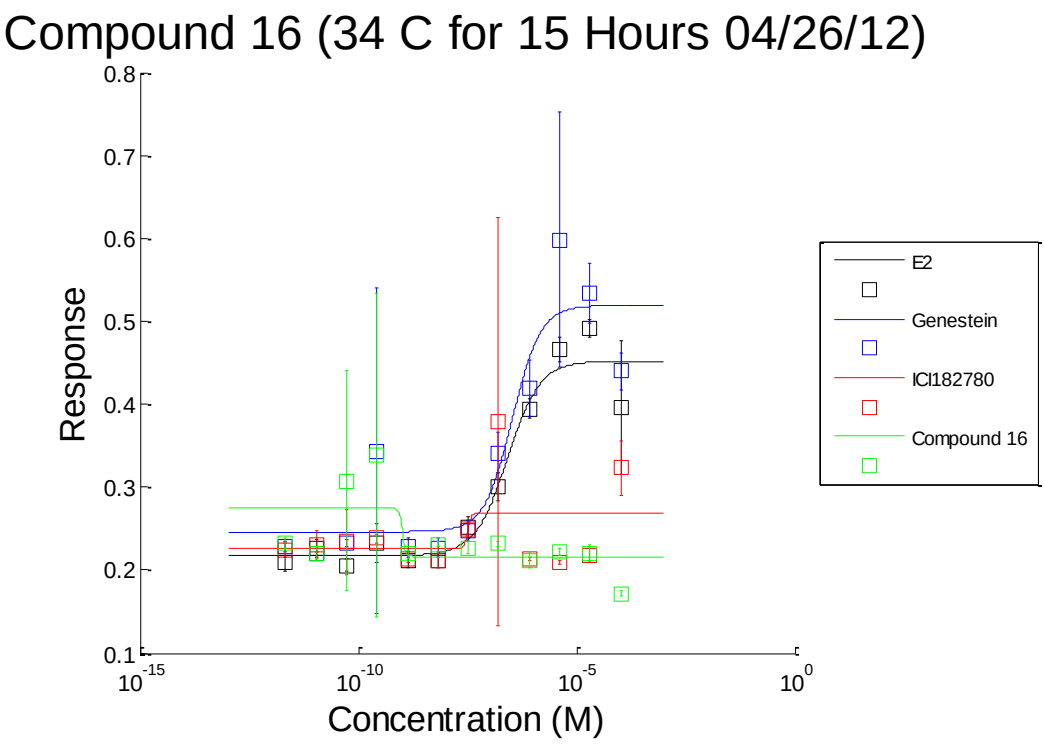

Figure 39: Dose-response curve for compound 16

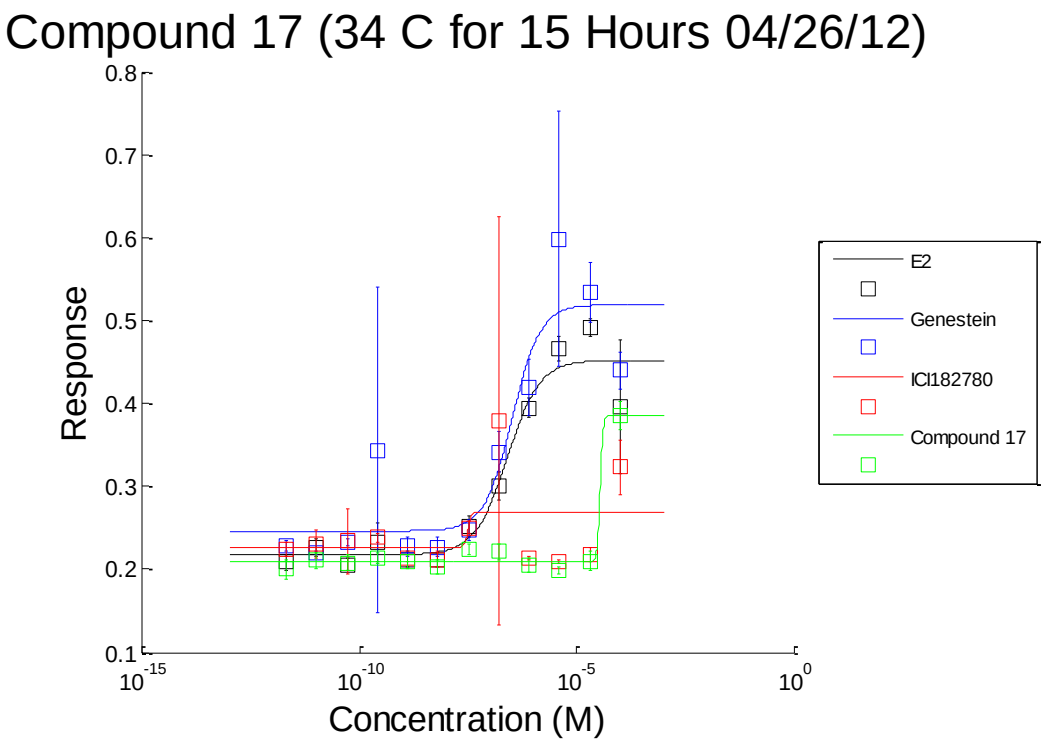

Figure 40: Dose-response curve for compound 17

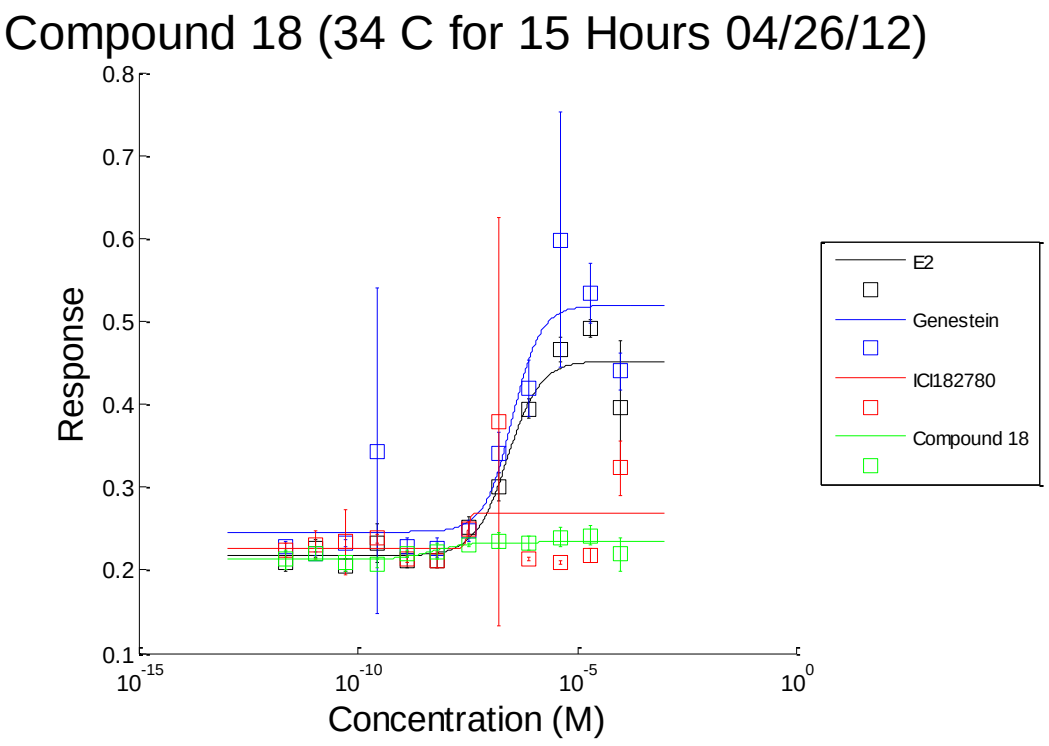

Figure 41: Dose-response curve for compound 18

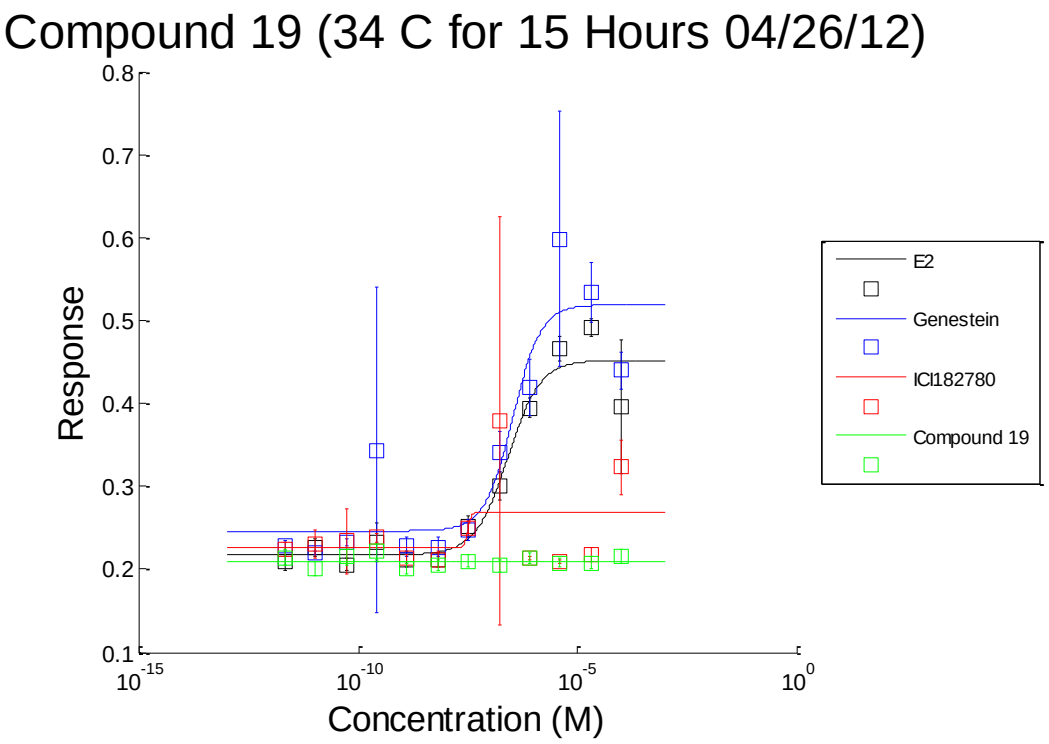

Figure 42: Dose-response curve for compound 19

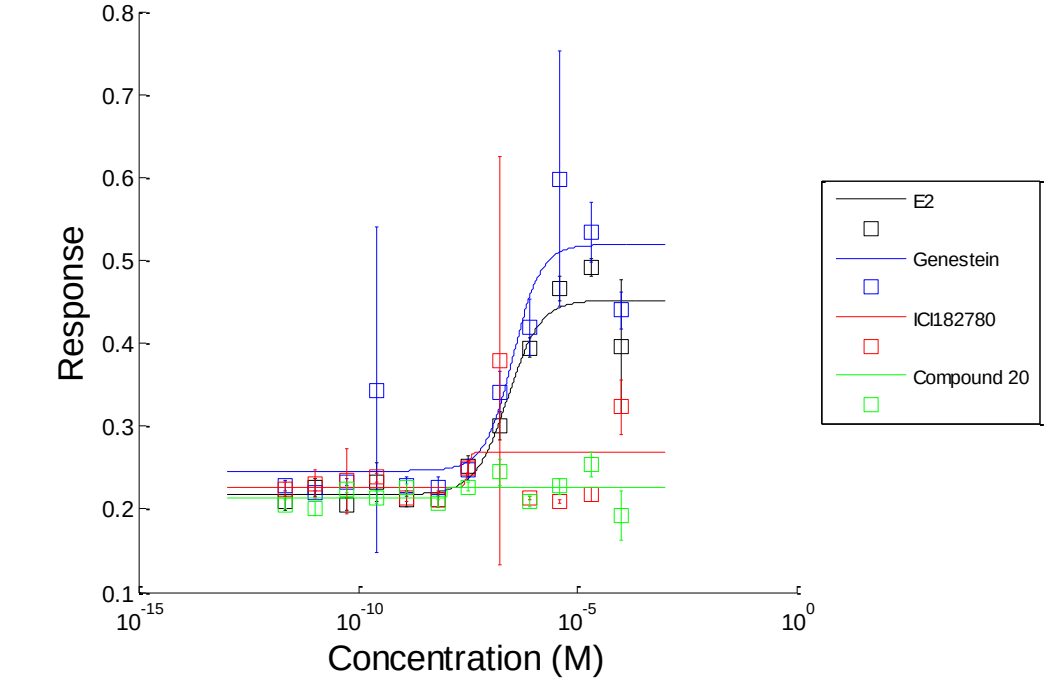

Figure 43: Dose-response curve for compound 20

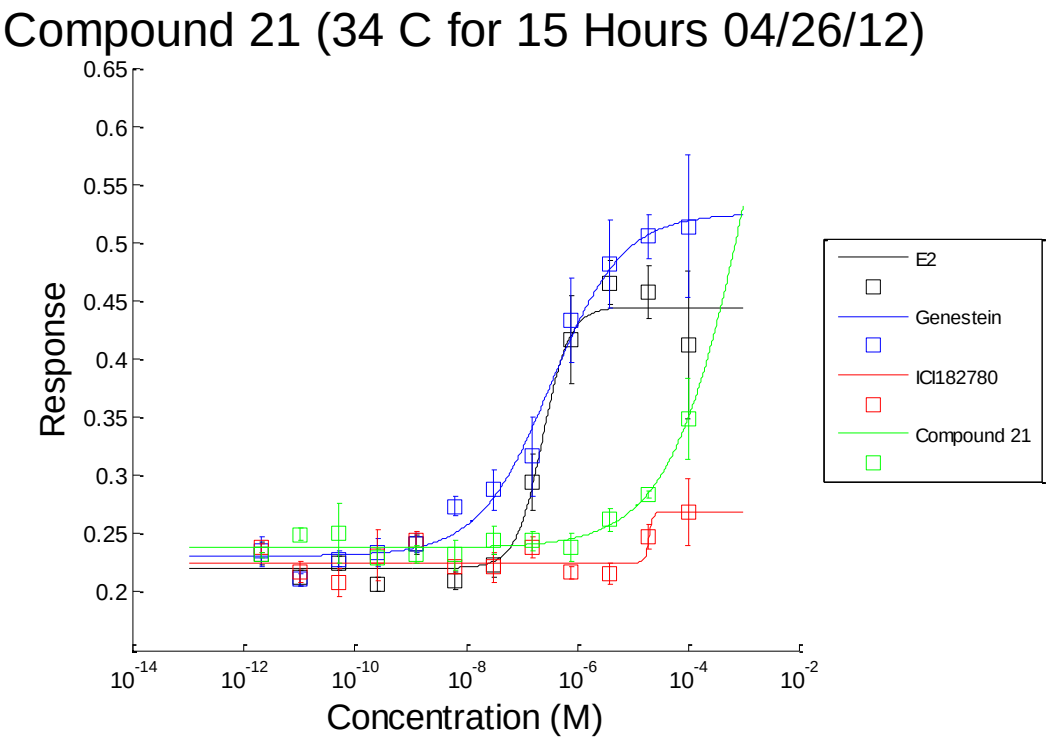

Figure 44: Dose-response curve for compound 21

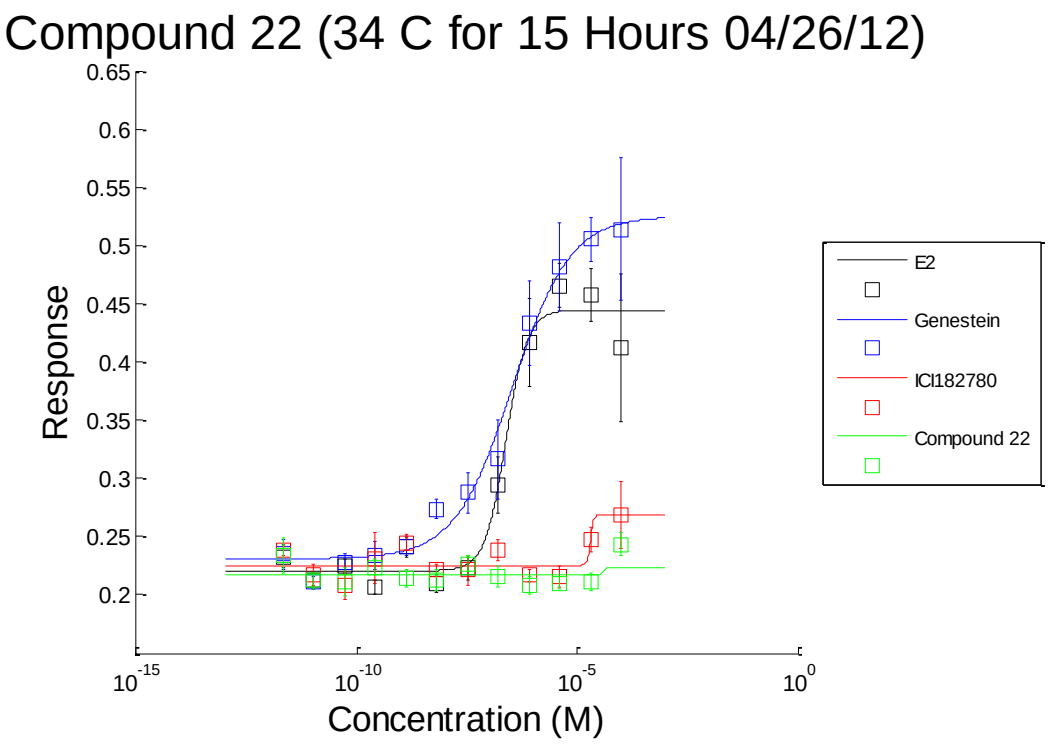

Figure 45: Dose-response curve for compound 22

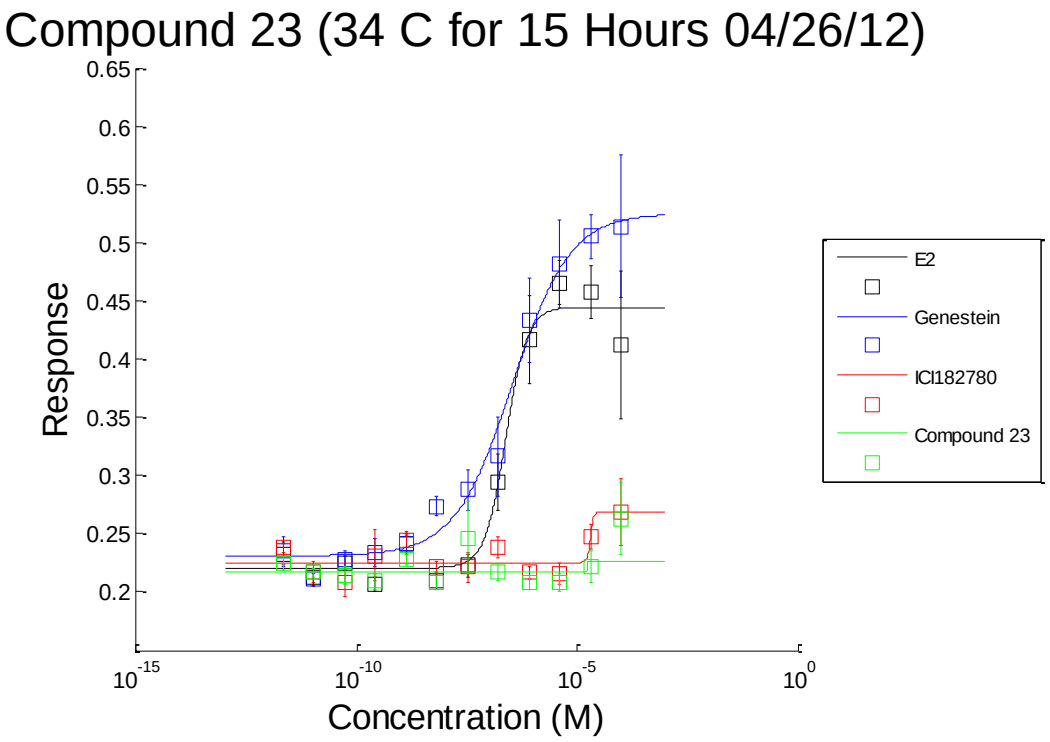

Figure 46: Dose-response curve for compound 23

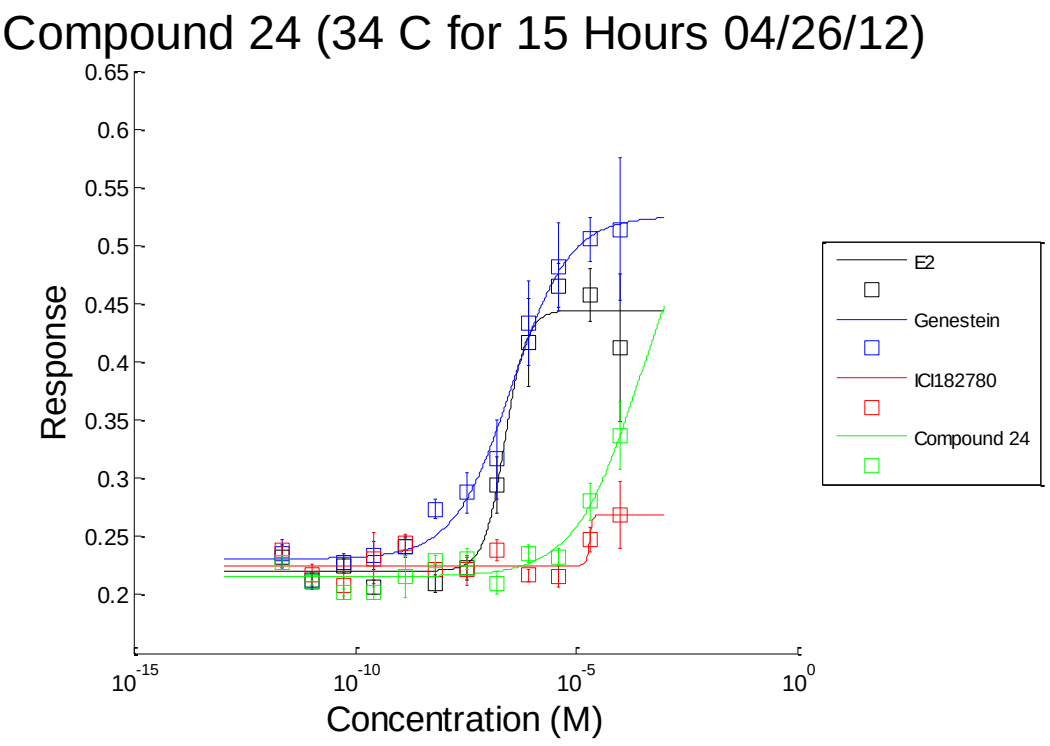

Figure 47: Dose-response curve for compound 24

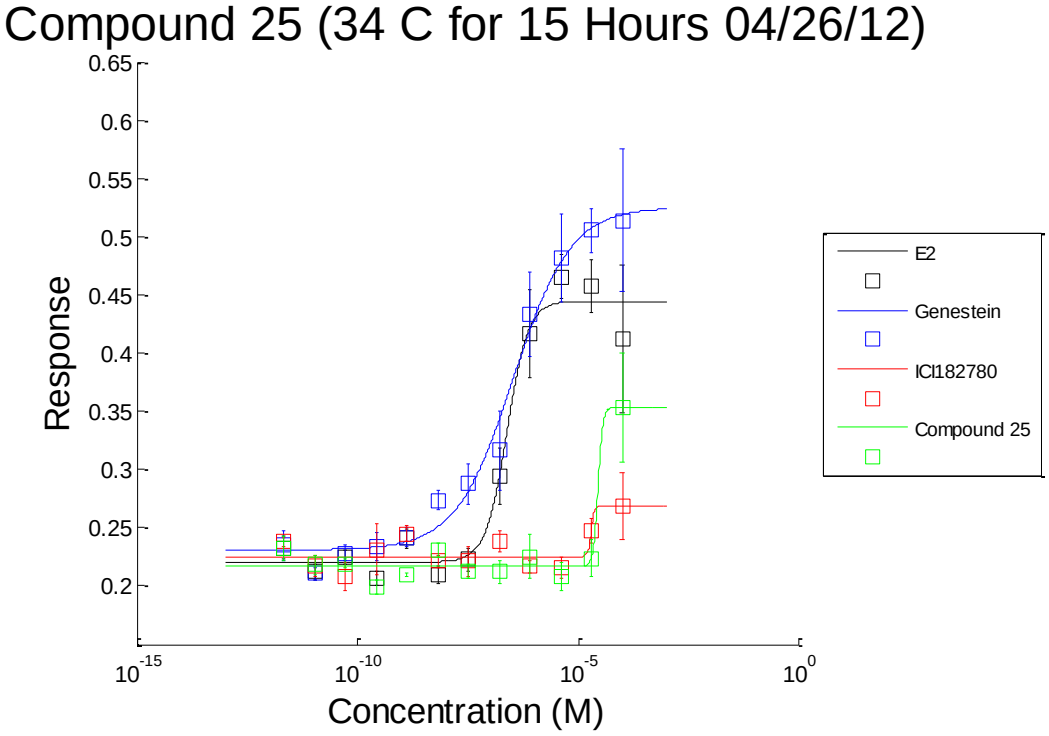

Figure 48: Dose-response curve for compound 25

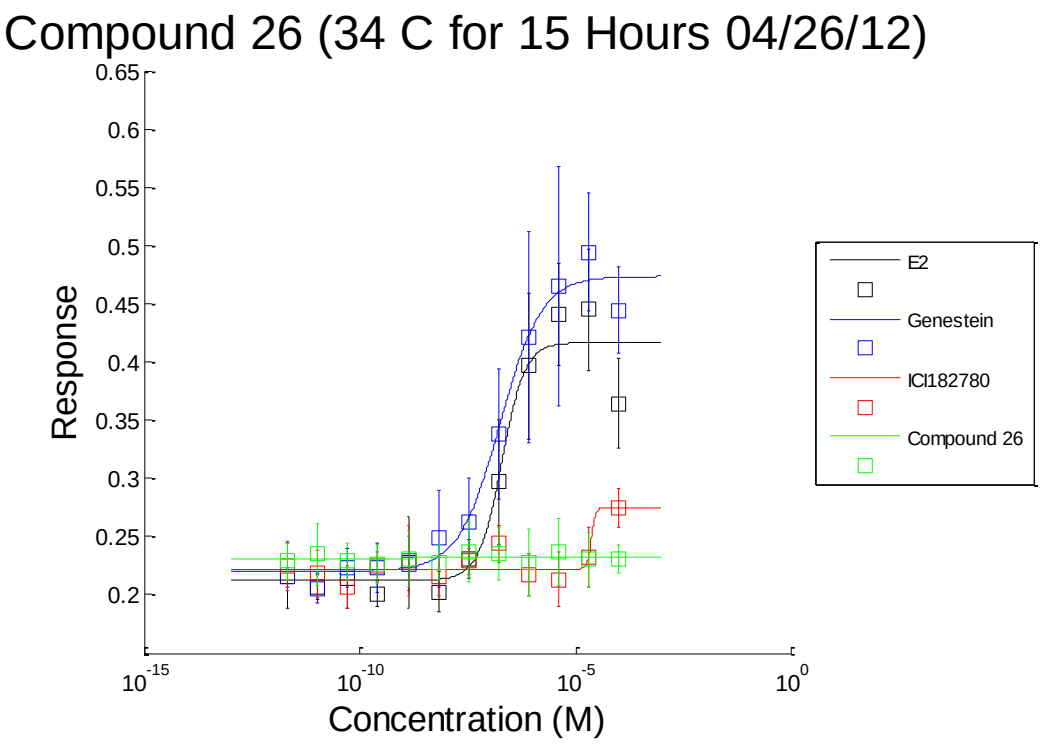

Figure 49: Dose-response curve for compound 26

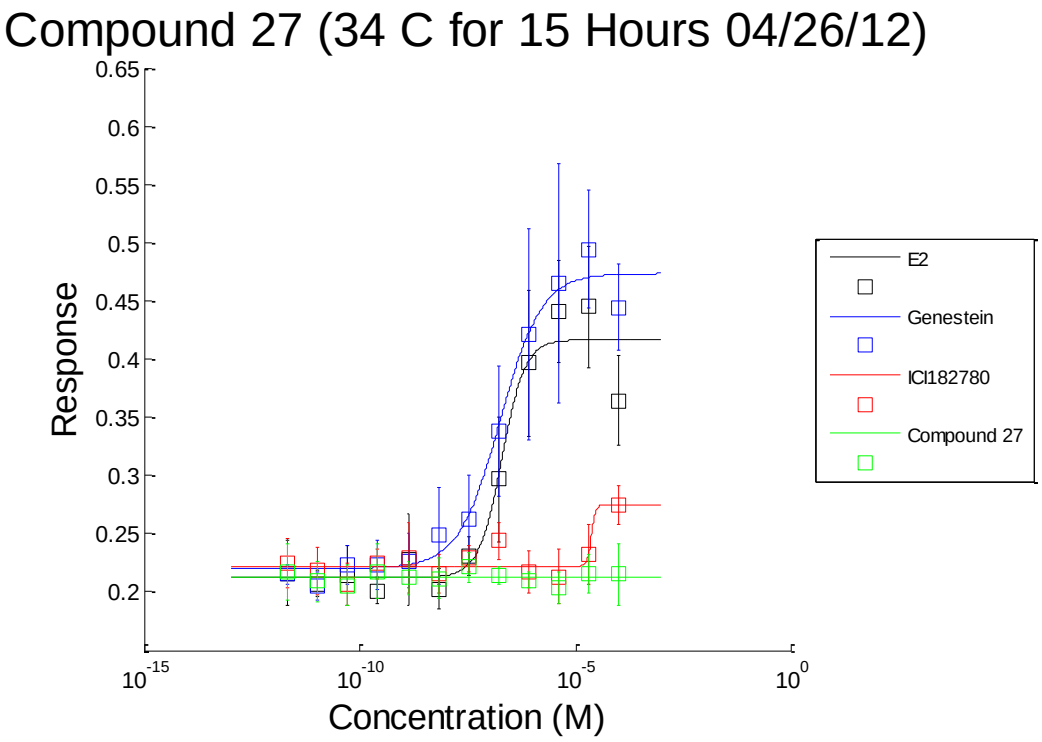

Figure 50: Dose-response curve for compound 27

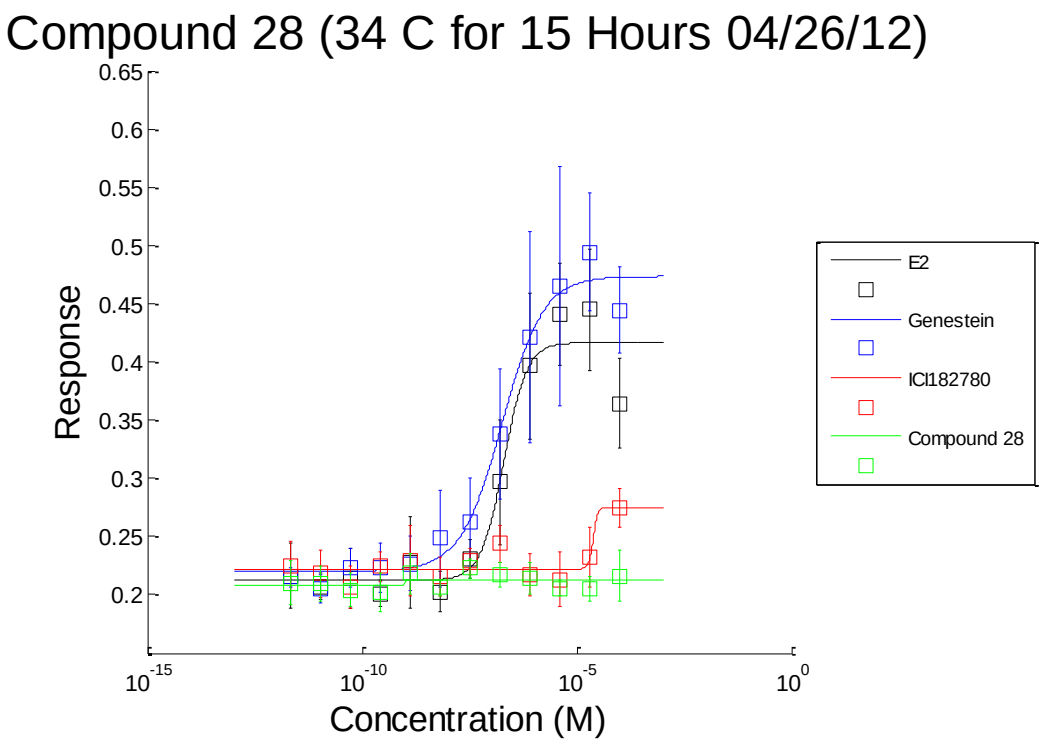

Figure 51: Dose-response curve for compound 28

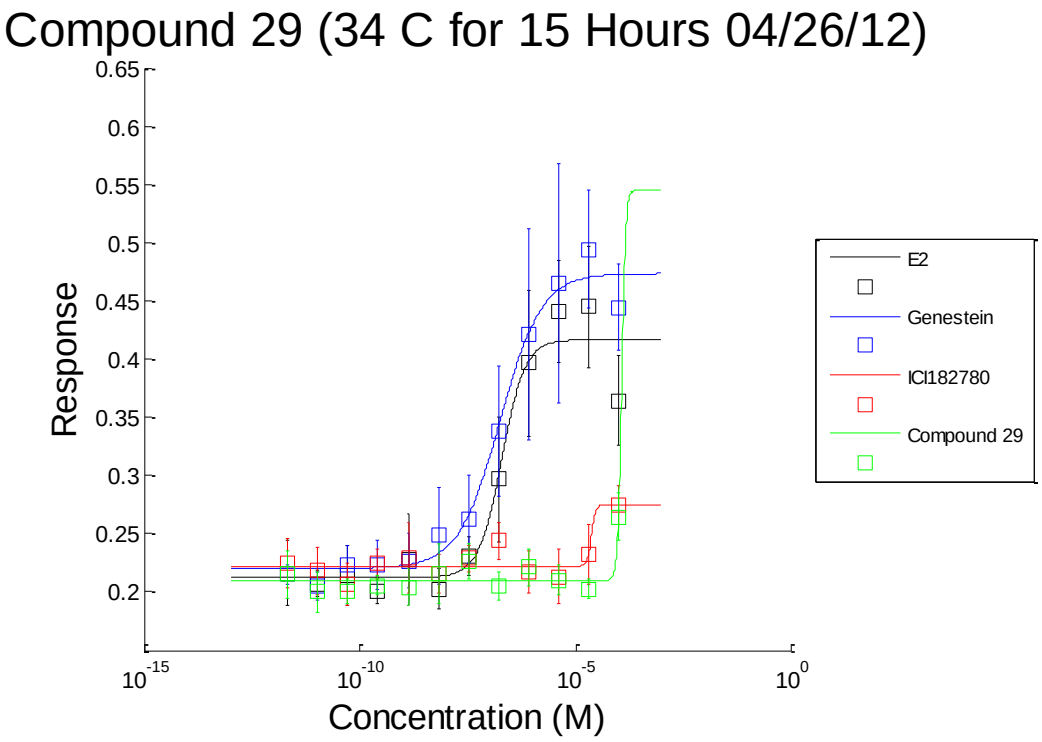

Figure 52: Dose-response curve for compound 29

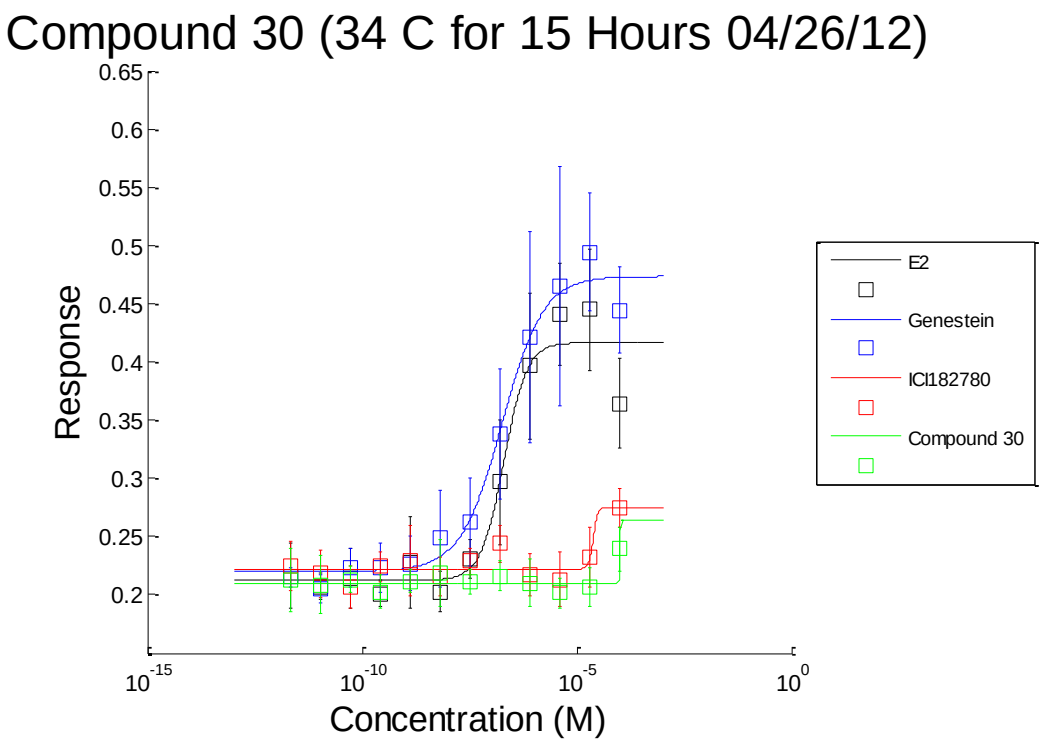

Figure 53: Dose-response curve for compound 30

Appendix F: Full Screening Results

Plate Number	Compound	Dose-Response Relationship Present	EC50 (M)	EC50 (μM)
1	Compound 1	No	N/A	N/A
	Compound 2	No	N/A	N/A
	Compound 3	No	N/A	N/A
	Compound 4	Yes	$6.92*10^{-9}$	$6.92*10^{-3}$
	Compound 5	No	N/A	N/A
	E2	Yes	$5.12*10^{-7}$	0.512
	Genestein	Yes	$4.90*10^{-7}$	0.490
	ICI 182,780	No	N/A	N/A
2	Compound 6	No	N/A	N/A
	Compound 7	Yes	$3.57*10^{-5}$	35.7
	Compound 8	No	N/A	N/A
	Compound 9	Yes	$4.91*10^{-5}$	49.1
	Compound 10	No	N/A	N/A
	E2	Yes	$1.94*10^{-8}$	0.0194
	Genestein	Yes	$2.81*10^{-7}$	0.281
	ICI 182,780	No	N/A	N/A
3	Compound 11	No	N/A	N/A
	Compound 12	No	N/A	N/A
	Compound 13	No	N/A	N/A
	Compound 14	No	N/A	N/A
	Compound 15	No	N/A	N/A
	E2	Yes	$1.84*10^{-7}$	0.184
	Genestein	Yes	$1.39*10^{-7}$	0.139
	ICI 182,780	No	N/A	N/A
4	Compound 16	No	N/A	N/A
	Compound 17	Yes	$3.50*10^{-5}$	35.0
	Compound 18	No	N/A	N/A
	Compound 19	No	N/A	N/A
	Compound 20	No	N/A	N/A
	E2	Yes	$2.54*10^{-7}$	0.254
	Genestein	Yes	$3.29*10^{-7}$	0.329
	ICI 182,780	No	N/A	N/A
5	Compound 21	Yes	0.002	2000
	Compound 22	No	N/A	N/A
	Compound 23	No	N/A	N/A
	Compound 24	Yes	$2.76*10^{-4}$	274
	Compound 25	Yes	$2.86*10^{-5}$	28.6
	E2	Yes	$2.41*10^{-7}$	0.241
	Genestein	Yes	$3.37*10^{-7}$	0.337
	ICI 182,780	No	N/A	N/A
6	Compound 26	No	N/A	N/A
	Compound 27	No	N/A	N/A

	Compound 28	No	N/A	N/A
	Compound 29	No	N/A	N/A
	Compound 30	No	N/A	N/A
	E2	Yes	$1.94*10^{-7}$	0.194
	Genestein	Yes	$1.76*10^{-7}$	0.176
	ICI 182,780	No	N/A	N/A